MEDICAL LIBRARY
AUG 4 1947

Vol. XXXVI July, 1947 No. 7

SCIENTIFIC EDITION

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

Justin L. Powers, Editor

Committee on Publications

George D. Beal, *Chairman*
B. V. Christensen
Roy B. Cook
Robert P. Fischelis
Hugo H. Schaefer

Editorial Advisory Board

L. W. Busse
T. C. Daniels
L. W. Hazleton
E. J. Hughes
W. T. Sumerford
E. H. Wirth

CONTENTS OF THIS ISSUE

Quantitative Assay of the Intravenous Toxicity of Streptomycin in Mice 193
Walther H. Ott

Trimethyl Cetyl Ammonium Pentachlorphenate (TCAP) and Fatty Acids as Antifungal Agents 198
E. J. Foley and S. W. Lee

Stability of Iodine Solutions and Tinctures 203
George D. Beal, Kenneth L. Waters, and Paul Block, Jr.

A Modified Kober Method for the Determination of Alpha- and Beta-Estradiol 208
Jonas Carol and J. C. Molitor

Availability of Penicillin from Various Ointment Bases 211
H. A. Clymer and R. J. Ferlauto

Some Minor Alkaloids of Pei-Mu, Fritillaria Roylei 215
T. Q. Chou

The Comparative Chronic Toxicities of Fumaric, Tartaric, Oxalic, and Maleic Acids 217
O. Garth Fitzhugh and Arthur A. Nelson

The Separation of Penicillins by Partition Chromatography 220
Henry Fischbach, Thomas E. Eble, and Merlin Mundell

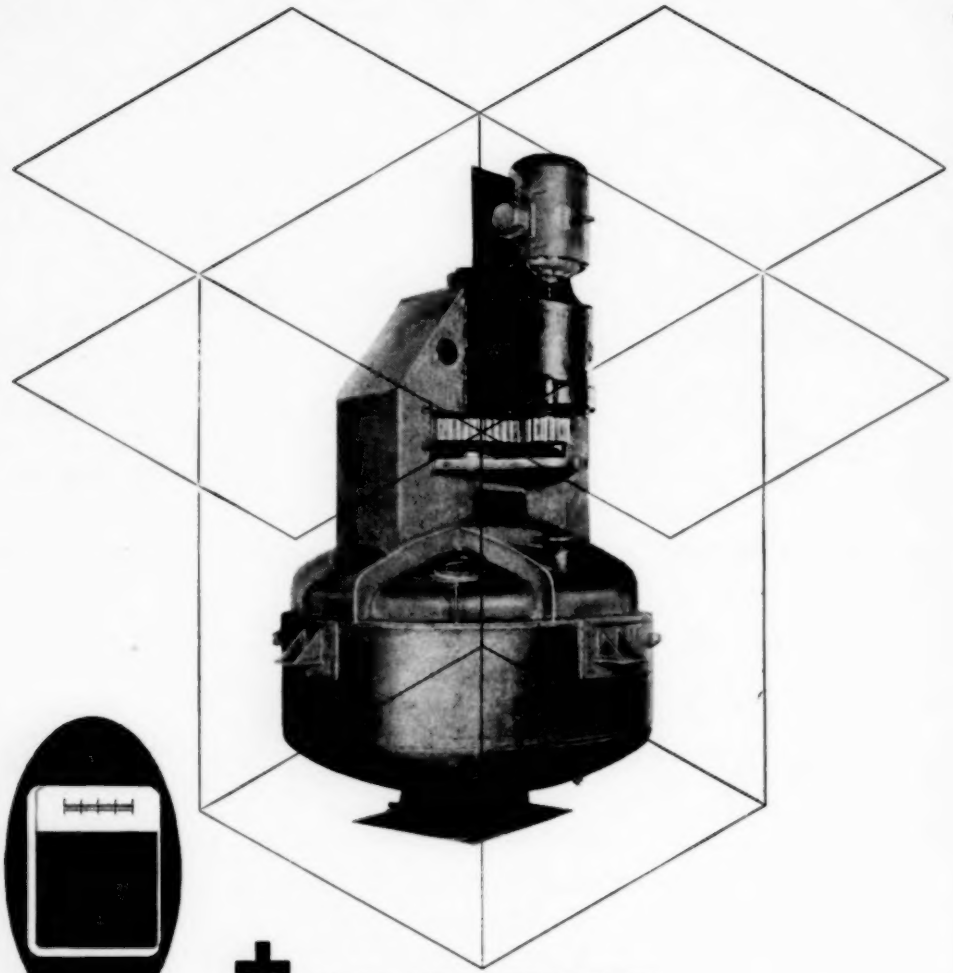
A Simple Color Reaction for Piperazine (A Note) 224
Robert D. Barnard

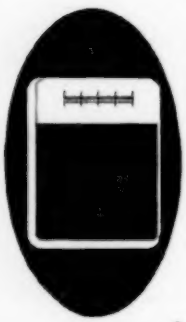
Book Review 223

Abstracts 193–224

Advertising ..... Cover pages 2, 3, 4 and pp. I–IV

Copyright, 1947, by the American Pharmaceutical Association





PILL TILE AND PRESSURE REACTOR

Surprising as it may seem, this huge pressure reactor is used in your prescription department more often than your old familiar pill tile.

We do not advise you, however, to install a pressure reactor in your store. It is a rather unwieldy piece of machinery—and quite expensive. A chemical plant is in a much better position to provide space for industrial equipment. Modern machines play, quite naturally, a vital part in the production of MALLINCKRODT PRESCRIPTION CHEMICALS. Modern machines help to create these fine substances that surpass U.S.P. standards for "pure" and meet the pharmacist's standards for "compoundable."

A pressure reactor may be, therefore, as indispensable to lege-artis-compounding as pill tiles or any of your other familiar equipment. Your skill and the medium of these tools produce the perfect preparation to which you proudly affix your prescription label.

A Few Mallinckrodt Prescription Chemicals

Acid Lactic	Magnesium Oxide	Magnesium Trisilicate	Potassium Acetate	Sodium Bromide
Ammonium Chloride	Hexamethylenamine	Phenobarbital	Silver Nitrate	Zinc Oxide

MALLINCKRODT CHEMICAL WORKS

80 YEARS OF SERVICE TO CHEMICAL USERS

MALLINCKRODT STREET, ST. LOUIS 7, MO. • 72 GOLD STREET, NEW YORK 8, N. Y.

PHILADELPHIA • CHICAGO • LOS ANGELES • MONTREAL

UNIFORM • DEPENDABLE • PURITY

Scientific Edition

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

JUSTIN L. POWERS, EDITOR, WASHINGTON, D. C.

VOLUME XXXVI · *JULY, 1947* · NUMBER 7
CONSECUTIVE NO. 14

Quantitative Assay of the Intravenous Toxicity of Streptomycin in Mice*

By WALTHER H. OTT

A method has been devised for the quantitative assay of the intravenous lethal toxicity of commercial streptomycin in mice whereby the LD_{50} is determined from the observed mortality by direct reference to tabulated values. Using certain specified doses and 5 mice per dose, two designs for the assay are presented: a two-dose method with a standard error of approximately ±8% of the LD_{50}, and a single-dose method with a standard error of ±10%.

THE NEED for a quantitative assay of the intravenous toxicity of commercial streptomycin in mice became apparent with the observation (1) that the intravenous toxicity in mice was unrelated to the potency of the drug and presumably was due to some substance, or substances, other than pure streptomycin. Although a safety test has been described by the Food and Drug Administration (2), this test shows only that the toxicity of the tested sample exceeds, or falls below, a certain limit approximately equivalent to an LD_0 of 1000 micrograms streptomycin base per 20-Gm. mouse. Therefore attention was directed toward development of a quantitative assay of reasonable accuracy using the technical specifications set forth by the F.D.A. for the safety test.

* Received March 7, 1947, from the Merck Institute for Therapeutic Research, Rahway, N. J.

MATERIALS AND METHODS

The mice used in these assays were of a uniform strain (CFI) obtained at weekly intervals from the Carworth Farms. The animals had continuous access to water and a nutritionally complete laboratory diet, and were housed in metal cages in quarters maintained at 75° F. Mice weighing from 19 to 24 Gm., inclusive, were used in all tests. The intravenous injection was made into a tail vein using a 1-ml. syringe with a 26 or 27 gauge needle. The injection time was kept as near as possible to five seconds with the aid of a metronome. All mice were observed continuously for thirty minutes, and the survivors were observed daily for two days. No mouse was ever used more than once.

In view of the fact that the experimental error of the median lethal dose, or LD_{50}, is less than that of any other lethal dose, especially the LD_0 (3), the LD_{50} was selected as the measure of toxicity. Biometrical methods described by Bliss, *et al.* (3, 4) were employed in analyzing and evaluating the data.

Using the above procedures, several experiments were conducted to study some of the factors influencing the test (Tables I and II). Five mice were used

on each dose in all but one of the experiments, in which 10 mice were used per dose. In each assay different doses were given until several were found that caused mortalities in the range between 0% and 100%. Within each comparison of relative toxicity (Table I) the various doses were prepared directly from the same stock solution of streptomycin. The factors other than the one being studied were kept constant from dose to dose within each assay. In addition to the factors listed, these included date of assay, age of mice, and individual injector.

Two strains of mice, CFW and CFl, were available, and they were found to differ significantly in resistance to the toxic properties of streptomycin. No explanation was sought for this difference. The CFl strain, being more readily available in large numbers, was adopted for all streptomycin toxicity assays in this laboratory.

Since mice differing by as much as 25% in body weight (usable range 19 to 24 Gm., inclusive) are employed in the assay, the relation between dose and body weight was investigated. When the dose of the drug was an amount calculated to be directly proportional to the body weight the toxicity of streptomycin was the same for the heaviest mice as for the lightest mice within the usable weight range. On the other hand, when the dose was kept constant regardless of body weight, an apparently lower toxicity value was obtained with the heavier mice. Therefore, in the conduct of the quantitative assay, the dose for each mouse was made proportional to the body weight by adjusting the volume of the prepared dose on the basis of 0.50 ml. per 20 Gm.

Four lots of streptomycin of different origins were assayed using approximately 10% increments in dose (Table II). Six doses, at least four of which gave mortalities between 0% and 100%, were used on each lot. Even though the preparations varied considerably in relative toxicity, the slopes of the four dosage-mortality curves did not differ significantly from parallelism ($\chi^2 = 1.5$, $df = 3$, $P = 0.66$). This observation is considered an indication that the toxic factors in the different materials produced qualitatively similar responses in the mice.

Although 5 mice seem to be a small number to use on each dose, this number has proved sufficient, presumably due in part to a high degree of uniformity among the mice. This was indicated by the fact that the observed mortalities for any of the four lots of streptomycin did not differ significantly from the composite curve (χ^2, Table II).

TABLE I.—INFLUENCE OF FOUR FACTORS ON THE ASSAY OF THE INTRAVENOUS TOXICITY OF STREPTOMYCIN IN MICE

No. Mice Used Per Dose	No. Mice Used Total	Assay Series		Mean Relative Toxicity[a] of Streptomycin (with Standard Errors)
5	195	Injection time, sec.:	5	100%
			10	102 ± 7.4%
			20	94 ± 7.1%
			30	83 ± 5.2%
			60	68 ± 4.3%
5	55	Sex:	Males	100%
			Females	93 ± 6.0%
5	125	Strain:	CFl	100%
			CFW	126 ± 5.4%
10	180	Injection dose:	per mouse, 19–20 Gm.	100%
			per mouse, 23–24 Gm.	87 ± 3.5%
			per 20 Gm., 23–24 Gm.	101 ± 4.1%

[a] The toxicity in one group within each series was arbitrarily assigned a value of 100%, and the toxicities in the other groups were expressed as a percentage of that value.

TABLE II.—INTRAVENOUS TOXICITIES OF STREPTOMYCIN OF DIFFERENT ORIGIN

Lot	No. Mice Used	LD_{50} Micrograms Base/20 Gm.	Individual Slope	Agreement between Observations and Fitted Curves[a] χ^2	Agreement between Observations and Fitted Curves[a] Probability
A	30	1440	13.1	3.5	0.45
B	30	1570	22.0	0.3	0.99
C	30	1130	19.2	2.7	0.60
D	30	2380	15.1	4.6	0.20

[a] Agreement is significant in each instance.

RESULTS

In making the transition from the slow rate of injection formerly used (1) to the rapid rate of 0.5 ml. in five seconds as adopted by the F.D.A. (2), it was found that the observed toxicity gradually increased as the injection time was shortened to about ten seconds (Table I). No further change in relative toxicity was apparent when the time was reduced to less than ten seconds.

The two sexes differed very slightly in sensitivity to the toxic factor, the females appearing to be more resistant than the males. Although this difference is not statistically significant and no difficulty has been encountered due to it both sexes have never been used on the same sample.

Data from the above assays and 10 routine assays selected at random were combined to obtain a satisfactory estimate of the slope of the curve relating logarithm of the streptomycin dose to per cent mortality in probits. As shown in Table III, the pooled slope for this entire series was calculated as 14.8. The individual assay curves were parallel within experimental error ($\chi^2 = 31$, $df = 45$), and consequently the slope of 14.8 has been used in the calculations in this paper and for the streptomycin toxicity assays conducted in this laboratory.

THE QUANTITATIVE ASSAY

Using the preceding information, the F.D.A. safety test was modified so as to systematize and standardize the procedure irrespective of the toxicity of the individual sample. The objective of the quantitative assay was the determination of the LD_{50} in a single assay with reasonable accuracy and without expenditure of unreasonable effort.

For these purposes certain selected doses were injected into mice, using 5 mice per dose, until two adjacent doses in the series were found such that two or fewer mice died at the lower dose and three or more mice died at the higher dose. The doses designated for use in this quantitative assay were spaced at approximately 20% increments and were as follows: 700, 850, 1000, 1200, 1450, 1750, 2100, 2500, and 3000 micrograms streptomycin base per 0.5 ml. (corresponding to values equally spaced on a logarithmic scale before rounding). No other doses were used routinely. The concentrations were given per 0.5 ml. because each dose was injected on the basis of 0.5 ml. of the prepared solution per 20 Gm. mouse. The selected doses were prepared as needed from a stock solution of the sample containing 3000 micrograms streptomycin base per 0.5 ml. Distilled water was used for all solutions.

Following injection of a lethal dose, death invariably occurred within thirty minutes, and usually within five minutes. In fact, among more than

TABLE III.—SUMMARY OF DATA FOR CALCULATION OF THE SLOPE OF THE DOSAGE-MORTALITY CURVE FOR TOXICITY OF COMMERCIAL STREPTOMYCIN IN MICE

Assay Series	No. of Curves	No. of Mice	[wx²]	[wxy]	Slope
Injection time	15	195	0.3212	4.8153	15.0
Sex	4	55	0.0916	1.2442	13.6
Strain (CFI)	4	65	0.0703	0.9311	13.2
Origin	4	120	0.1291	2.2252	17.2
Injection dose	9	180	0.1477	2.2023	14.9
Routines	10	150	0.2307	3.2573	14.1
All	46	765	0.9906	14.6754	14.8

TABLE IV.—QUANTITATIVE TWO-DOSE ASSAY OF STREPTOMYCIN TOXICITY IN MICE[a]

Dose pair		No. of mice dead out of 5 mice injected per dose				
		3	3 4	3 4 5	4 5	5
High	Low	0	1 or 0	2 or 1 or 0	2 or 1	2
850	700	850	810	770	740	700
1000	850	1020	970	920	880	840
1200	1000	1210	1150	1100	1050	990
1450	1200	1450	1380	1320	1260	1200
1750	1450	1760	1670	1590	1520	1440
2100	1750	2110	2010	1920	1830	1740
2500	2100	2530	2400	2290	2190	2080
3000	2500	3020	2870	2740	2610	2480

[a] LD_{50}'s in the body of the table correspond to the 9 possible mortality combinations in which 0, 1, or 2 mice died at the lower dose and 3, 4, or 5 mice died at the higher dose in each of the 8 dose pairs. An individual assay is completed when any of these mortality combinations are obtained with any one of the 8 dose pairs.

All values are micrograms base per 20 Gm. mouse.

Example: The 2100-microgram dose is injected, killing 3 out of 5 mice. The next lower dose must then be given, which is 1750 micrograms. This dose kills 1 out of 5 mice. The mortality combination is 3 at 2100 and 1 at 1750, $\binom{3}{1}$, which is found at the heading for the second mortality column. Opposite the 2100-1750 dose pair is found 2010, the LD_{50} corresponding to these results.

The inherent precision of this assay is relatively high ($1/b = 0.068$) compared to other all-or-none assays (5). For the 10 routine assays (Table III) 2 to 4 doses and an average of 15 mice were used per sample. The average standard error of the LD_{50} was calculated as $\pm 6.3\%$ of the LD_{50}, a value indicative of satisfactory reproducibility for this assay.

In order to insure that known and unknown factors would not interfere beyond the limits of experimental error with the day to day accuracy of routine assay results, a sample of commercial streptomycin was assayed daily as a working standard. Statistical quality control (6, 7) was maintained on the LD_{50} of this standard as a measure of the resistance of the different groups of mice used from day to day. Confidence limits were established at the 95% probability values (7), and samples were considered to have been assayed satisfactorily when the LD_{50} of the standard fell within these limits.

10,000 mice surviving this initial observation period, only 10 deaths in the following two days (all from one sample) were observed. No deaths occurred among approximately 2500 surviving mice which were observed for ten days after the injection. Accordingly, the mortality occurring in the initial observation period can be considered the final value for each dose, and the assay can be conducted from dose to dose without appreciable delay until the requirements for the design of the assay have been met.

The LD_{50} may then be read from Table IV as the value corresponding to the particular mortalities and pair of doses in each assay. This table was prepared for the 9 possible mortality combinations which can be obtained for each of the 8 dose pairs fulfilling the requirements set forth above for the quantitative assay. The LD_{50}'s in the table were computed for the corresponding pair of doses and mortalities using the slope of 14.8 and the methods described by Bliss (3).

Since the high precision of this assay is not required for many types of routine samples, the assay was simplified for such samples by designing a one-dose procedure. This was accomplished by omitting alternate levels in the series of doses employed in the two-dose assay, and by requriing that one of these doses be found which kills 1, 2, 3, or 4 out of 5 mice or that two adjacent doses in this particular series of doses (Table V) be found such that no mice die at the lower dose and all mice die at the higher dose.

DISCUSSION

With the assay using two doses and 5 mice per dose the average standard error of the LD_{50} was calculated as approximately $\pm 8\%$. However, by running a duplicate assay on a sample the standard error of the average LD_{50} is reduced to approximately $\pm 6\%$. In actual practice a standard error of $\pm 4.2\%$ of the average LD_{50} was found in duplicate assays conducted in the above manner on 50 samples during a period of almost three months. In this group of assays, duplicate values were obtained on 7 samples, and none of the differences between LD_{50}'s in duplicate assays on the remaining samples exceeded 18.8% of the average LD_{50} in each instance. Therefore, the actual reproducibility of the assay appears to be slightly better than that expected from the theoretical standard error of $\pm 6\%$.

The standard error of the LD_{50} in the one-dose assay was calculated as approximately $\pm 10\%$. Although a large number of samples have been assayed using the one-dose method, relatively few of the samples were reassayed because the agreement between duplicate assays has been very satisfactory. Among 10 samples reassayed in a four-month period, duplicate values were obtained on three samples, while a difference of 33% between LD_{50}'s in duplicate assays was found on one and differences ranging from 4% to 16% on the remaining six samples. This reproducibility is somewhat better than expected, as was also noted with the two-dose assay.

TABLE V.—QUANTITATIVE ONE-DOSE ASSAY OF STREPTOMYCIN TOXICITY IN MICE[a]

Dose	No. of Mice Dead Out of 5 Mice Injected				
	1	2	3	4	0[b]———5[b]
700	800	730	670	610	x (580−)
1000	1150	1050	960	880	840 x x
1450	1650	1500	1400	1250	1200 x x
2100	2400	2200	2000	1850	1750 x x
3000	3400	3100	2900	2650	2550 (3600+) x

[a] LD_{50}'s in the body of the table correspond to 4 mortalities possible with each dose and to one mortality combination possible with a pair of adjacent doses.
All values are micrograms base per 20 Gm. mouse.
Example: The 1450-microgram dose is injected, killing no mice. A higher dose must be given. The 2100 dose is chosen which kills 3 out of 5 mice, thus completing the assay. Opposite the 2100 dose in the column for 3 dead is found 2000, the LD_{50} corresponding to this result. If all 5 mice had died at the 2100 dose, the LD_{50} would be found in the 0———5 column as 1750.
[b] None dead at one dose and all dead at next higher dose.

The occurrence of any one of these 5 possible mortalities completes this one-dose assay, and the corresponding LD_{50} is found by reference to Table V. In other respects this assay is performed in the same manner as the two-dose assay. Only doses selected from the 5 doses listed in Table V are used in this assay. The LD_{50}'s in this table were computed by means of the equation, $X = x + \frac{(5 - y)}{14.8}$, where X is the log LD_{50} corresponding to the per cent mortality in probits, y, and to the log dose, x (3).

A minimum of 10 mice is needed for a single two-dose assay (5 mice per dose) and 20 mice for a duplicate two-dose assay. On the other hand, the minimum for the one-dose assay is 5 mice. However, it can be seen that not more than 15 mice (3 doses) need ever be used in the one-dose assay provided the 1450 microgram dose is the first dose administered. In actual routine use, an average of 7 mice has been used per sample with the one-dose method, and an average of 11 mice has been used per sample with the two-dose method. These results were accomplished with samples of relatively uniform, but unknown, toxicity ranging from 700 to 3000 micrograms streptomycin base per 20 Gm. mouse.

It is to be noted that quantitative results were obtained by the above assay methods on samples of unknown toxicity with the average expenditure of not more than twice the time and effort required for the purely qualitative safety test, namely 7 to 11 mice per sample for the quantitative assays as compared to 5 mice per sample for the safety test. The advantages of having available the LD_{50} of every sample have far outweighed the disadvantages of the extra work involved. Furthermore, the procedure for the quantitative assay has been designed so that the assay is as easy to conduct and evaluate as the safety test itself.

The LD_{50}'s in the tables for the quantitative assays (Tables IV and V) were computed for a dosage-mortality curve having a slope of 14.8, and are valid particularly for the assay of intravenous toxicity of commercial streptomycin in mice. As a matter of fact, the above tables could be used in the conduct and evaluation of assays of the toxicity of any drug exhibiting a slope of approximately 14.8 for the dosage-mortality curve. Since the slope of the dosage-mortality curve may vary from laboratory to laboratory, it is recommended that each laboratory determine the slope of the curve, and if it differs significantly from 14.8, the principles applied above be used to design similar tables for use under their particular conditions.

SUMMARY

1. An assay method of relatively high precision has been developed for the quantitative determination of the intravenous toxicity of commercial streptomycin in mice using certain specified doses and 5 mice per dose, and evaluating the LD_{50} from the resulting mortalities by direct reference to tabulated values.

2. Two designs for the assay are described: a two-dose assay with a standard error of approximately $\pm 8\%$ and a one-dose assay with a standard error of about $\pm 10\%$.

3. In routine assays using the two-dose assay, an average of 11 mice has been used per sample. Using the one-dose assay the average has been 7 mice per sample.

4. The slope of the dosage-mortality curve for this assay was estimated at 14.8.

5. The observed toxicity of streptomycin increased as the length of the injection period was shortened from one minute to ten seconds. No difference was found between the ten-second and five-second rates.

6. Females were not significantly different from males in resistance to the toxicity of streptomycin.

7. A significant difference in resistance was observed between the two strains of mice that were investigated.

8. The observed toxicity of streptomycin was relatively constant when doses of the drug were given in proportion to body weight in the range from 19 to 24 Gm.

9. No qualitative differences in toxicity were detected among four lots of streptomycin of different origins.

REFERENCES

(1) Molitor, H., Graessle, O. E., Kuna, S., Mushett, C. W., and Silber, R. H., *J. Pharmacol.*, **86**, 151–73(1946).

(2) Food and Drug Administration, Washington, D. C., "Specifications for Streptomycin. Standards of identity, strength, quality and purity. Nov. 20, 1945, and July 15, 1946." (Mimeographed reports).

(3) Bliss, C. I., *Quart. J. Pharm. Pharmacol.*, **11**, 192–216 (1938).

(4) Miller, L. C., Bliss, C. I., and Braun, H. A., THIS JOURNAL, **28**, 644–57(1939).

(5) Bliss, C. I., and Cattell, M., *Ann. Rev. Physiol.*, **5**, 479–539(1943).

(6) Knudsen, L. R., and Randall, W. A., *J. Bact.*, **50**, 187–200(1945).

(7) Bliss, C. I., *Biometrics Bull.*, **1**, 57–65(1945).

Trimethyl Cetyl Ammonium Pentachlorphenate (TCAP) and Fatty Acids as Antifungal Agents*

By E. J. FOLEY and S. W. LEE

The antifungal activity of an experimental ointment and two solutions containing trimethyl cetyl ammonium pentachlorphenate was determined and compared with results obtained by similar methods using preparations containing fatty acids or their salts.

THE STUDIES of many authors (1–8, 15, 16, 24, 25) have established that fatty acids have marked antifungal properties, and therapeutic preparations of considerable merit containing fatty acids or fatty acid salts have recently been used for the treatment of dermatophytoses (9–12, 14) and tinea of the scalp (13).

In previous publications (15, 16) we have shown that trimethyl cetyl ammonium pentachlorphenate (TCAP) has marked fungicidal properties and that free fatty acids are far more effective *in vitro* against pathogenic fungi than are the salts of these acids. Of the higher fatty acids studied (heptylic, caprylic, pelargonic, capric, and undecylenic) the last appeared to be the most powerful antifungal agent. Appropriate experiments clearly indicated that by the use of vehicles which solubilize undecylenic acid or TCAP, their activity was greatly enhanced.

In order to obtain superior fungicidal action, it was advantageous to use the 2 agents, TCAP and undecylenic acid, in combination (24), and proceeding from the above facts, experiments were made to determine the best means of providing optimum conditions for fungicidal effect. *In vitro* tests were devised to study the antifungal action of these agents alone and in combinations in various vehicles under different experimental conditions. In addition, comparisons were made with products containing fatty acids and fatty acid salts which have been recommended for the treatment of dermatophytoses. The present paper describes the results obtained and includes toxicity data on 3 preparations which have been used in extensive clinical trials in the treatment of various forms of tinea.

The fungicidal efficiency of TCAP is penetrant vehicles against *Microsporon audouini* has been described in a preliminary report by MacKee, *et al.* (13). These authors found that TCAP in an "Intraderm" base was the best fungicidal agent among a large number examined when tests were conducted with infected hairs from cases of tinea capitis. The clinical results obtained with this preparation were gratifying and the course of treatment was well tolerated.

MATERIALS AND METHODS

Liquid culture medium was made with 1% Nutripeptone (Baltimore Biological Laboratories) and 4% maltose. The agar media consisted of this broth plus 2% agar. Three methods of *in vitro* assay have been used to test the antifungal action of the preparations under study.

1. Fungistatic Tests (Agar or Broth Dilution Method).—A modification of the Kolmer method in which 0.1 ml. of dilutions of the drugs in water is added to 4.9 ml. of melted agar or broth. The *p*H of the media was adjusted to 5.0 and 7.0 by the addition of MacIllvaine's buffers after sterilizing. Spore suspensions were prepared by washing 1 ten-day agar slant culture of *Trichophyton gypseum* (American Type Culture Collection #9533) *Trichophyton purpureum, M. audouini, Microsporon lanosum, Epidermophyton inguinale,* or *Monilia albicans* in 15 ml. of saline. In the tests 0.01 ml. of the spore suspensions was added to each tube. The results were recorded after two weeks' incubation at 30°. Inhibitory concentrations are expressed as final dilutions per ml. of agar. We have expressed the results obtained by this method as "fungistatic action," although transoculation experiments have proved that the TCAP preparations are fungicidal in all but the highest dilutions.

2. Fungistatic Tests (Diffusion Zone Method in Agar).—Ten milliliters of agar inoculated with 0.01 ml. of a heavy spore suspension (*T. gypseum*) is poured into a Petri dish and allowed to harden. Porcelain tops are used to minimize condensation. When liquids are to be tested, 1-cm. filter paper circles are dipped in the preparations, drained, and placed on the surface of the agar. Ointments are placed on the surface of the inoculated agar in 1-cm. circles. Inhibition zones are measured after four days' incubation at 30°.

* Received Oct. 7, 1946, from Wallace Laboratories, Inc., New Brunswick, N. J.

3. Fungicidal Tests.—The fungicidal action of liquids was determined by the Emmons (17) and by the Burlingame–Reddish methods (18), using 10-ml. tubes of 1% neopeptone, 2% dextrose broth adjusted to *p*H 7.0 by the addition of sterile phosphate buffer. *T. gypseum* ATCC #9533 was used as the test organism. A special procedure, which we have called the "agar mixture technique," was devised to determine the fungicidal action of ointments. Five-tenths milliliter of the preparation under test was mixed with 9.0 ml. melted Sabouraud's agar (Difco) *p*H 5.6 in a test tube which was then cooled to 45°. The agar was poured into a Petri dish containing 0.5 ml. of a dilution of *T. gypseum* spores, mixed thoroughly, and made to harden quickly by holding the plate for two minutes on a chilled surface. The inoculum was adjusted so that the addition of 0.5 ml. to 9.5 ml. of the agar-drug mixture gave one million spores per ml. of the final agar mixture. Plugs 0.5 cm. in diameter were removed from the agar with a sterile cork borer and seeded into 10 ml. of broth adjusted to *p*H 7 at ten-, twenty-, and thirty-minute intervals after the plates were poured. Results were recorded after two weeks' incubation at 30°.

Composition of Experimental Antifungal Preparations

Experimental ointments were prepared by conventional methods using a partly esterified mixture of fatty acids, principally stearic, but containing oleic and palmitic acids and higher alcohols, as the "wax" component.

The basic formula to which the various active ingredients were added was as follows:

Wax	21.0 Gm.
Spermaceti	3.3 Gm.
Propylene glycol N. F.	5.5 Gm.
Water *q. s.* to make	100.0 Gm.

In order to facilitate discussion and to illustrate the formulation of the experimental ointments in general Formula "A" is given as an example:

Formula "A"

Trimethyl cetyl ammonium pentachlorphenate (40% aqueous slurry)	5.5 (2.2%)
Undecylenic acid	1.0
Wax	21.0
Spermaceti	3.3
Propylene Glycol N. F.	5.5
Water (Distilled)	63.7

Variations of this formula made for comparisons are indicated in Table I.

The solutions studied were variations of the preparation described by MacKee, *et al.* Formula "B" is a fungicidal solution containing 3.2% TCAP, 1% undecylenic acid, 10.8% propylene glycol, 9.8% sulfonated castor oil, 12.6% antipyrine, a wetting agent of the alkyl benzene sulfonate type 5.2%, and water.

Formula "C" is a fungicidal solution containing 1% TCAP, 1% undecylenic acid, 12% propylene glycol, 12% sulfonated castor oil, 3.5% antipyrine, a wetting agent of the alkyl benzene sulfonate type 6%, and water.

All three of the preparations tested had a *p*H of about 5.0.

EXPERIMENTAL

A variety of experimental preparations were compared for antifungal activity by the agar dilution technique using *T. gypseum* as the test organism. The results are shown in Table I.

TABLE I.—ANTIFUNGAL ACTION OF VARIOUS PREPARATIONS DILUTED IN WATER AND INCORPORATED IN SABOURAUD AGAR AT *p*H 5.6 AND *p*H 7 AGAINST *T. gypseum*

	*p*H 5.6	*p*H 7
5% propionic ointment	<1/500[a]	<1/500
1% undecylenic ointment	1/1000	<1/500
5% undecylenic ointment	1/8000[b]	<1/500
5% caprylic ointment	1/3000	<1/500
2.2% TCAP ointment	1/4000[c]	1/1500
(Formula A) 2.2% TCAP + 1% undecylenic ointment	1/4000	1/1500
2.2% TCAP + 5% undecylenic ointment	1/8000[c]	1/1500
2.2% TCAP + 5% caprylic ointment	1/4000	1/1500
TCAP liquid (Formula B) 3.2% TCAP + 1% undecylenic acid	1/8000[d]	1/2500
TCAP liquid (Formula C) 1% TCAP + 1% undecylenic acid	1/3000	1/1000
TCAP Liquid 3.2% TCAP 1% propionic acid (Formula B except propionic replacing undecylenic acid)	1/8000	1/2500
Zinc undecylenate 20% / Undecylenic acid 5% } ointment	1/1500	<1/500
Sodium propionate 16.4% / Propionic acid 3.6% } ointment	<1/500	<1/1500

[a] The figures shown indicate the dilutions of ointment per ml. of agar preventing the growth of *T. gypseum*.
[b] Subcultures of agar show fungicidal action at a dilution of 1/2000.
[c] Subcultures of agar show fungicidal action at a dilution of 1/3000.
[d] Subcultures of agar show fungicidal action at a dilution of 1/5000.

The data in Table I show that the antifungal properties of the various preparations are a function of the concentration of the ingredients and of their behavior at *p*H 5.6 or *p*H 7.

Propionic acid and sodium propionate are known to be weak fungistatic agents and ointments containing large amounts of these ingredients were not inhibitory at a 1/500 dilution. The 1% and 5% undecylenic acid ointments were active at *p*H 5 roughly in proportion to concentration of the acid. The 5% caprylic acid ointment was weaker than the 5% undecylenic acid ointment. The zinc undecylenate ointment was significantly less active than the

5% undecylenic acid ointment. The 2.2% TCAP ointment inhibited when diluted 1/4000. The addition of 1% undecylenic acid or 5% caprylic acid to this formula did not increase its inhibitory action. However, when 5% undecylenic acid was added it inhibited at a dilution of 1/8000. It is of importance to compare the degree of inhibition obtained at *p*H 5 with those obtained at *p*H 7. It will be noted that at the higher *p*H, the preparations containing TCAP were still active at a dilution of 1/1500 or higher, while those containing fatty acids or salts of fatty acids failed to inhibit at the 1/500 dilution.

The activity of the TCAP ointments at *p*H 7 is, therefore, an expression of the antifungal properties of the quaternary ammonium salt. Furthermore, it will be noted that the addition of 1% undecylenic or 5% caprylic acid to ointments containing 2.2% TCAP does not increase their antifungal properties over that of an ointment containing 2.2% TCAP only. When 5% undecylenic acid is added to the 2.2% TCAP ointment, however, the inhibitory titer rises to 1/8000.

The inhibitory titer exhibited by the TCAP liquids at both *p*H 5 and *p*H 7 are what would be expected on the basis of the amounts of TCAP present. It is obvious that the TCAP-undecylenic acid preparations, as exemplified by Formulas A B, and C, have marked antifungal properties.

parative activity of Formulas A, B, and C, when tested by the dilution method in agar and broth. The results of such a comparison are shown in Table II.

The data in Table II show that an even higher range of activity is manifest by preparations A, B, and C when tested in broth than when tested in agar dilutions. It is probable that the lower fungistatic end points obtained in agar, as compared with those obtained in broth, are due to inhibition of TCAP by agar. However, as previously noted, agents other than quaternary ammonium compounds give higher values when tested against pathogenic fungi in broth than when tested in agar.

Growth inhibition tests were also carried out against *T. purpureum*, *M. audouini*, *M. lanosum*, *E. inguinale*, and *Monilia albicans* by the agar dilution technique. The results are shown in Table III.

The data in Table III show that Formulas A, B, and C are highly active against pathogenic fungi other than *T. gypseum*. All 3 preparations inhibit *T. purpureum*, *M. audouini*, *M. lanosum*, and *E. inguinale* in high dilution when tested at *p*H 5.6.

The preparations are less active when tested at *p*H 7, yet even at this *p*H considerable activity is retained. *Monilia albicans* is more resistant to the drugs than are the fungi.

The inhibiting action of several of the preparations

TABLE II.—ANTIFUNGAL ACTION OF VARIOUS PREPARATIONS DILUTED IN WATER AND INCORPORATED IN AGAR OR BROTH MEDIUM AT *p*H 5.6 and *p*H 7 AGAINST *T. gypseum*

	*p*H 5.6		*p*H 7.0	
	Agar	Broth	Agar	Broth
Formula A, TCAP ointment 2.2% TCAP, 1% undecylenic acid	1/4000	1/6000	1/1500	1/2000
Formula B, TCAP liquid 3.2% TCAP, 1% undecylenic acid	1/8000	1/12,000	1/2500	1/4500
Formula C, TCAP liquid 1% TCAP, 1% undecylenic acid	1/3000	1/4500	1/1000	1/2000

TABLE III.—FUNGISTATIC ACTION OF TCAP-UNDECYLENIC ACID OINTMENT AND LIQUIDS AGAINST VARIOUS PATHOGENIC FUNGI AND *Monilia Albicans*

	Dilutions of Preparations Inhibiting Growth in Agar at *p*H 5.6 and *p*H 7.0					
	Formula A		Formula B		Formula C	
	Agar *p*H 5.6	Agar *p*H 7.0	Agar *p*H 5.6	Agar *p*H 7.0	Agar *p*H 5.6	Agar *p*H 7.0
T. purpureum	1/4000	1/800	1/10,000	1/1000	1/5000	1/800
M. audouini	1/5000	1/800	1/10,000	1/1000	1/5000	1/800
M. lanosum	1/5000	1/800	1/5000	1/800	1/5000	1/600
E. inguinale	1/5000	1/800	1/16,000	1/1000	1/5000	1/800
Monilia albicans	1/400	1/500	1/500	1/400	1/400	1/400

It has previously been shown that higher end points are obtained when broth is used to test the fungistatic action of fatty acids than when tests are made in agar medium. We have confirmed this observation (24) using the fatty acids and have shown that TCAP is more active when tested in broth than when tested in agar. Recently Quisno, *et al.* (27) have shown that the action of quaternary ammonium salts are impaired in the presence of agar. It was of importance to determine the com-

in Table I, as indicated by the growth inhibition zone technique, are shown in Table IV.

It has been previously noted that the width of the inhibitory zones produced by such agents as the higher fatty acids and TCAP is influenced by their solubility (15, 16). This being the case, indications of the comparative antifungal activity of these materials can only be obtained if serial dilutions in suitable solvents are compared (24). When the data in Table IV are compared with those in Table

TABLE IV. —INHIBITION OF *T. Gypseum* BY VARIOUS ANTIFUNGAL AGENTS DIFFUSING THROUGH SABOURAUD'S AGAR *p*H 5.6

	Cm.
2.2% TCAP ointment	3.8
2.2% TCAP + 1% undecylenic acid ointment (Formula A)	4.2
2.2% TCAP ointment + 5% undecylenic acid	4.5
Zinc undecylenate 20% / Undecylenic acid 5% } Ointment	4.5
Sodium propionate 16.4% / Propionic acid 3.6% } Ointment	5.4
Liquid (Formula C), 1% TCAP, 1% undecylenic acid (Formula C)	4.8
Liquid (Formula B), 3.2% TCAP, 1% undecylenic acid (Formula B)	5.2

I, it is apparent that no true gauge of the comparative activity of ointments containing water-insoluble antifungal agents can be obtained by merely placing ointments on a culture plate, according to the commonly used inhibition zone technique.

In Table IV, the sodium propionate ointment produced the widest zone of inhibition, obviously because the active ingredient is water soluble. The zone of inhibition produced by this ointment is wider than that produced by the 3.2% TCAP, 1% undecylenic acid solution, yet, the propionate ointment failed to inhibit growth when diluted 1/500 (Table I), while the TCAP-undecylenic acid solution was inhibitory at a dilution of 1/8000 and was proved by subculturing to the fungicidal at a dilution of 1/5000. The fungicidal effects of TCAP solutions were tested by the Emmons technique (17) and by the Burlingame–Reddish technique (18). The results are shown in Table V.

technique. These results are shown in Table V.

It will be seen in Table V that the marked fungicidal activity of the TCAP solutions is demonstrated by the Emmons and the agar mixture technique. It is of interest also to note the fungicidal capacities of the ointments as shown by the agar mixture technique. One to 20 dilutions of the 2.2% TCAP, 1% undecylenic acid ointment, and the zinc undecylenate ointments killed *T. gypseum* spores within ten minutes, but the sodium propionate ointment failed to kill within the thirty-minute exposure period. It was noted above that agar may have an adverse effect on the activity of TCAP, and for this reason the agar mixture fungicidal technique cannot be claimed to give critical quantitative values. In our hands, however, the method has given useful data, and we regard it as an additional tool in studying antifungal drug preparations.

Numerous acute and chronic toxicity studies on TCAP in the Intraderm base were made in animals and sensitization studies were made in rats, rabbits, and guinea pigs repeatedly inuncted with the solution. In addition, large numbers of patch tests and cumulative inunction tests were made in humans to determine primary irritation or hypersensitivity to Intraderm TCAP and TCAP Ointment.

No evidence of primary irritation or sensitization or of chronic toxicity was noted in the inuncted animals, and no instance of irritation or sensitization occurred in a series of 330 human subjects repeatedly inuncted with the products and retested after a two-week interval. The acute toxicity of a 2.5% TCAP solution in water was determined on mice. The results, expressed in terms of % survival five

TABLE V.—FUNGICIDAL ACTION OF VARIOUS PRODUCTS AS SHOWN BY SEVERAL METHODS OF TESTING

	Technique by Which Tests Were Conducted		
	Emmons	Burlingame–Reddish	Agar Mixture
Formula B	Killed 10 min.	Killed 30 min.	Killed 10 min.
Formula C	Killed 10 min.	Not killed 30 min.	Killed 10 min.
Formula A			Killed 10 min.
Zinc Undecylenate 20% / Undecylenic Acid 5% } Ointment			Killed 10 min.
Sodium propionate 16.4% / Propionic acid 3.6% } Ointment			Not killed 30 min.
Sodium propionate 16.4% / Propionic acid 3.6% } Liquid	Not killed 30 min.	Not killed 30 min.	Not killed 30 min.

Rapid killing of spores (within ten minutes) was demonstrated by the Emmons method; this result is in keeping with the result of MacKee, *et al.* However, no killing was detected within thirty minutes by the Burlingame–Reddish method.

In order to study the killing effects of the liquids under conditions which would assure contact of the active ingredients with the spores, a special technique, described under Methods and called the "agar mixture technique," was devised. It was feasible to study the fungicidal capacities of ointments by this

days after the subcutaneous injection of the solution are shown in Table VI.

TABLE VI.—ACUTE TOXICITY OF 2.5% AQUEOUS TCAP SOLUTION (MICE)

Subcutaneous Injections, Ml./Kg.	Survival after 5 Days, Per Cent
20	30
15	60
10	100

DISCUSSION

The data presented emphasize the facts previously noted by us that solubilized undecylenic acid and TCAP have remarkable fungicidal properties and indicate that their use in combination according to either Formulas A, B, or C is warranted because of the high order of activity shown *in vitro* (Table I) and because of the absence of contraindications on grounds of toxicity.

We have previously noted (15, 16, 24) the superiority of free fatty acids over the corresponding salts in antifungal activity and have pointed out that TCAP is similarly influenced by *p*H although to a lesser extent since considerable activity is shown by this compound at *p*H 7 and *p*H 8. It should be emphasized that the products containing the combination of active ingredients are extremely active at the *p*H of the normal glabrous skin and scalp and retain much activity even at higher *p*H such as may be encountered in infected areas or in the places where sweat evaporation is retarded (26).

The vehicles described have been prepared on the basis of compatibility with the active ingredients. Particular attention has been given to obtaining a mildly acid *p*H because of superior activity in the range of *p*H of the normal skin.

The use of solubilizing vehicles is important in obtaining the best results (15, 16, 24). It is obvious that water-insoluble active ingredients must be incorporated in suitable solubilizing vehicles if maximum effects are to be obtained. In the case of the TCAP, undecylenic acid ointment (Formula A) the wax combination used forms a solubilizing emulsion in which the active ingredients are dispersed. The liquid vehicles (Formulas B and C) have specialized properties. Formula B has well-defined skin penetrating action (19–23) and has been prepared for the treatment of intractable fungous infections, especially those in which the fungus may be thought to reside below the skin surface.

Formula C contains 1% TCAP and 1% undecylenic acid in true solution and has a high degree of fungicidal action (Table I). It was prepared for the treatment of, and as a prophylactic against, dermatophytoses of the glabrous skin, and as a prophylactic shampoo against tinea capitis.

The data in Table I show that the preparations containing TCAP and undecylenic acid have powerful antifungal action. The ointment (Formula A) containing 2.2% TCAP and 1% undecylenic acid capable of inhibiting the growth of *T. gypseum* when diluted 1/4000 in agar media at *p*H 5.6, the Intraderm liquid (Formula B) containing 3.2% TCAP inhibits at a dilution of 1/8000, and the fungicidal liquid (Formula C) containing 1% each of TCAP and undecylenic acid inhibits at a dilution of 1/3000. Still higher ranges of activity are obtained when tests are carried out in broth, and it is probable that the figures obtained by methods involving the use of agar culture media fail to reveal the true antifungal activity of the products containing TCAP.

A high order of activity is manifest by all 3 preparations at *p*H 7.0. Furthermore, as can be seen in Table IV, they have a rapid fungicidal action. Another point of interest in regard to the physical charactristics of the preparations is seen in Table III, in which a satisfactory degree of diffusion of the active ingredients in agar media is demonstrated.

SUMMARY AND CONCLUSIONS

The antifungal activity of 3 preparations containing trimethyl cetyl ammonium pentachlorphenate (TCAP) and undecylenic acid has been compared with other preparations containing fatty acids or their salts, some of which have been reported to be useful for the treatment of dermatophytosis.

The TCAP-undecylenic acid preparations were found to be more active against pathogenic fungi than were the other preparations studied.

The effect of solubilizing vehicles and *p*H of the preparations on the activity of fatty acids and TCAP has been discussed.

REFERENCES

(1) Marchionini, A., and Cerutti, P., *Arch. Dermatol. Syphilis*, **166,** 354(1932).
(2) Herrmann, F., and Fuerst, L., *Dermatol. Wochschr.*, **88,** 397(1929).
(3) Hoffman, D., Schweitzer, T. B., and Dolby, G., *Food Research*, **4,** 539(1939).
(4) Peck, S. M., and Rosenfeld, H., *J. Investigative Dermatol.*, **4,** 237(1938).
(5) Peck, S. M., Rosenfeld, H., Leifer, W., and Bierman, W., *Arch. Dermatol. Syphilol.*, **39,** 126(1939).
(6) Reigler, N. E., and Greathouse, G. A., *Am. J. Botany*, **27,** 701(1940).
(7) Kenny, E. L., *Bull. Johns Hopkins Hosp.*, **83,** 379 (1943).
(8) Hillegas, A. B., and Camp, E., *J. Investigative Dermatol.*, **6,** 217(1945).
(9) Kenney, E. L., and Broyles, E. N., *Bull. Johns Hopkins Hosp.*, **83,** 479(1943).
(10) Keeney, E. L., Libero, A., Broyles, E. N., and Lankford, E., *ibid.*, **75,** 417(1944).
(11) Keeney, E. L., Libero, A., Lankford, E., and Mary, L., *ibid.*, **77,** 422(1945).
(12) Sulzberger, M. B., Shaw, H. C., and Kanof, A., *U. S. Naval Med. Bull.*, **45,** 237(1945).
(13) MacKee, G. M., Herrmann, F., and Karp, I. L., *J. Investigative Dermatol.*, **7,** 43(1946).
(14) Hopkins, J. G., Fisher, J. K., Hillegas, A. B., Ledin, B. R., Rebell, G. C., and Camp, E., *ibid.*, **7,** 239(1946).
(15) Foley, E. J., Herrmann, F., and Lee, S. W., *ibid.*, in press.
(16) *Ibid.*, in press.
(17) Emmons, C. W., *Am. J. Pub. Health*, **35,** 844(1945).
(18) Burlingame, E. M., and Reddish, G., *J. Lab. Clin. Med.*, **24,** 765(1939).
(19) MacKee, G. M., Sulzberger, M. B., Herrmann, F., and Baer, R. L., *J. Investigative Dermatol.*, **6,** 43(1945).
(20) MacKee, G. M., Herrmann, F., Baer, R. L., and Sulzberger, M. B., *J. Lab. Clin. Med.*, **28,** 1642(1943).
(21) MacKee, G. M., Wachtel, J., and Karp, F. L., *J. Investigative Dermatol.*, **5,** 308(1945).
(22) Herrmann, F., Sulzberger, M. B., and Baer, R. L., *Science*, **96,** 451(1942).
(23) Herrmann, F., Sulzberger, M. B., and Baer, R. L., *N. Y. State J. Med.*, **44,** 2452(1944).
(24) Lee, S. W., and Foley, E. J., Personal observations; to be published.
(25) Wyss, O., Ludwig, B. J., and Joiner, R. T., *Arch. Biochem.*, **7,** 415(1945).
(26) Herrmann, F., Behrendt, H., and Karp, F., *J. Investigative Dermatol.*, **7,** 215(1946).
(27) Quisno, R. A., Gibby, I. W., and Foter, M. J., THIS JOURNAL, **35,** 317(1946).

Stability of Iodine Solutions and Tinctures*,†,‡

By GEORGE D. BEAL§, KENNETH L. WATERS‖, and PAUL BLOCK, JR.¶

Storage experiments on Iodine Tincture U. S. P., Strong Iodine Tincture N. F., Solution of Iodine N. F., and Strong Solution of Iodine U. S. P. have been carried out over a period of thirty months. These preparations were found to be stable in clear, amber, and blue glass-stoppered bottles. Additional studies on Iodine Tincture when in actual use in first-aid cabinets for a period of a year showed that this preparation maintained the required Pharmacopœia strength. Assay of collected samples of Strong Iodine Tincture which had been subject to household use for indefinite periods indicate that improperly stored samples of this product may have an iodine content higher than that permitted by the official compendia.

ALTHOUGH a tincture of iodine was not recognized in the first Pharmacopœia of the United States, we find that both of the 1830 revisions (1, 2) contain Tincture of Iodine. The Pharmacopœia of 1840 (3) recognized not only Tincture of Iodine prepared by dissolving 1 oz. of iodine in a pint of alcohol, but also a Compound Tincture and a Compound Solution of Iodine. The Compound Solution was prepared by dissolving 6 drachms of iodine and $1^1/_2$ oz. of potassium iodide in a pint of water. This preparation has remained virtually unchanged during the past century and appears in the Thirteenth Revision (4) as Strong Solution of Iodine. The Compound Tincture contained $^1/_2$ oz. of iodine and 1 oz. of potassium iodide in a pint of alcohol, which is approximately half the strength of Strong Tincture of Iodine N. F. VIII (5). In the 1850 revision (6) the Compound Tincture was deleted. The status of the approximately 7 per cent tincture remained relatively unchanged until 1905 at which time the Eighth Revision (7) directed that 5 per cent of potassium iodide be added. U. S. P. IX (8) stated that 50 cc. of water should also be added. This preparation remained in the Pharmacopœia until the Thirteenth Revision at which time it was deleted and is now official in National Formulary VIII (5).

A 2 per cent tincture was added to U. S. P. XI (9) as an antiseptic solution for extemporaneous skin sterilization and for use as a first aid dressing for cuts and wounds in the home. This Mild Tincture was introduced after extensive investigation (10, 11) had shown its general usefulness and bactericidal efficiency. The milder tincture does not appear to destroy tissues thus promoting more rapid healing than does the 7 per cent tincture. In addition, the 2 per cent tincture is prepared with dilute alcohol rendering it considerably less irritating when applied to open wounds. The Pharmacopœial Subcommittee on Scope recognized the advantages of the 2 per cent tincture and voted to delete the 7 per cent tincture and to change the name of Mild Tincture of Iodine U. S. P. XII to Iodine Tincture U. S. P. XIII. Realizing the usefulness and popularity of the stronger tincture the National Formulary VIII (5) adopted the 7 per cent tincture.

Karns, Cretcher, and Beal (12) have demonstrated that protein absorbs iodine more readily from an aqueous solution than from an alcoholic solution of equal strength. Further studies indicate that the dosage deposited depends not only on the solvent, but also on the iodide to iodine ratio. It has been found by adjusting this ratio that a solution could be obtained which would deposit an adequate dosage yet contain a smaller amount of iodine. A 2 per cent aqueous solution proved satisfactory in

* Received April 25, 1947, from the Department of Research in Pure Chemistry, Mellon Institute, Pittsburgh, Pa.
† This research was done with the assistance of a grant from the Board of Trustees of the United States Pharmacopœial Convention and with cooperation of the Iodine Fellowship at Mellon Institute.
‡ The names of tinctures and solutions used in this paper are in conformance with the new titles adopted for U. S. P. XIII. The familiar 7% Tincture of Iodine has been deleted from the U. S. P. and is official as Strong Iodine Tincture N. F. VIII. Mild Tincture of Iodine U. S. P. XII is known as Iodine Tincture U. S. P. XIII. Solution of Iodine has been deleted from the U. S. P. and is official as Iodine Solution N. F. VIII. Strong Iodine Solution (Lugol's solution) remains in the U. S. P.
§ Assistant Director, Mellon Institute.
‖ Former Fellow, Department of Research in Pure Chemistry, Mellon Institute.
¶ Former Industrial Fellow, Mellon Institute.

this respect. In such a solution the chemical and osmotic contributions to pain are considerably lessened, thereby giving a more physiologically desirable preparation.

A water solution of iodine is superior to an alcoholic solution in rate of penetration (13) and is just as efficient as a bactericide (14). The clinical test of Secor (15) showed the 2 per cent aqueous solution to be an excellent antiseptic for general hospital use. Consequently, Solution of Iodine was made official in the U. S. P. XII (16). However, this product did not gain popular acceptance, possibly because of the low rate of evaporation of the aqueous solvent and the higher freezing point, and was deleted from U. S. P. XIII. It is official under the name Iodine Solution in N. F. VIII.

The stability of tinctures and solutions of iodine has been questioned on many occasions since they were first introduced into the Pharmacopœia. Investigators have claimed that the changes which take place have been due to the glass (17), the impurities in the alcohol (18), the closures (19), heat (20), and light (21). Potassium iodide was used in the preparation of Solution of Iodine and in Compound Tincture of Iodine in the Pharmacopœia of 1840 (3). It is probable that the addition was for purposes of solubility rather than stability but writers soon pointed out the fact that potassium iodide increased the keeping qualities (20, 22, 23). In 1916 Gianturco (20) stated that six months was the maximum safe period for keeping iodine solutions intended for chirurgical purposes. In 1928 Bohrish (21) claimed that the preparations are stable but are kept best in completely filled containers of brown glass. Others (18, 24, 25) have also shown that the preparations are relatively stable.

Since the Thirteenth Revision of the Pharmacopœia (4) recognizes one tincture and one solution and the National Formulary (5) recognizes one tincture and one solution, it was thought it would be interesting to study the keeping qualities of these preparations. We have stored Iodine Tincture U. S. P., Strong Iodine Solution U. S. P., Strong Iodine Tincture, N. F., and Iodine Solution N. F. under various conditions and in clear, amber, and blue glass-stoppered bottles, the light transmission of which has been measured. Our observations are that even when stored under drastic conditions, such as exposure to direct sunlight during summer months, the preparations are relatively stable. The free iodine content of the official preparations showed little change during the thirty-month storage. Measurements of acidity showed that the tinctures after storage ranged from *p*H 3.8 to *p*H 4.7 whereas the aqueous solution measured *p*H 7.0 to *p*H 7.5. Apparently no appreciable acidity is developed during storage.

It is realized that placing the preparations in glass-stoppered bottles and opening them periodically to remove samples for analysis may not be typical of the conditions to which such preparations are subjected in actual use. Since some manufacturers have claimed that the 2 per cent tincture does not maintain its strength when in use, we have filled 2-oz. clear, glass-stoppered bottles with tincture of iodine and placed the bottles in first-aid cabinets where they were in constant use for one year. The use of glass-stoppered bottles eliminated the possibility of reaction with the closure and insured compliance with U. S. P. packaging requirements in that the container shall not interact physically or chemically with the drug it holds.

The re-analysis of these samples after one year showed that there was very little change although in some cases only a few cubic centimeters of tincture remained in the bottle. There was a slight increase in the per cent of free iodine, but none of the samples rose above the permissible maximum limit of 2.2 per cent.

To investigate further the practical keeping qualities of iodine preparations we collected bottles of Strong Iodine Tincture from individuals. These samples had no known history other than that they were purchased by the individual as Tincture of Iodine U. S. P., some of them probably five years previous to our collection. Since no previous analysis had been made by us it can only be assumed that these samples were within the limits for free iodine when

purchased. Our analysis showed that 8 of the 13 samples collected had an iodine content greater than 7.5 per cent, the maximum permitted for the official product. Some of the closures on the samples containing the higher per cent of iodine were faulty, which could account for increase through evaporation of solvent.

Our experiments indicate that the official preparations of iodine are relatively stable when properly stored, i.e., with closures resistant to iodine. The results of the analysis of the household samples of the Strong Iodine Tincture showed that this preparation in actual home use apparently loses alcohol by evaporation, thus increasing its iodine content. Iodine Tincture showed little increase in iodine content when in use in first-aid cabinets during the course of one year, indicating that if this product is stored properly it is a stable preparation.

EXPERIMENTAL

Storage of Official Preparations in Clear, Amber, and Blue Glass-Stoppered Bottles

Iodine Tincture, Strong Iodine Tincture, Iodine Solution, and Strong Iodine Solution were made in accordance with directions given in the United States Pharmacopœia XIII (4) and in the National Formulary VIII (5). The preparations were placed in 2-L., glass-stoppered bottles and allowed to age for a period of three months in the dark. They were then transferred to clear, amber, and blue, glass-stoppered bottles. A detailed description[1] of these bottles follows:

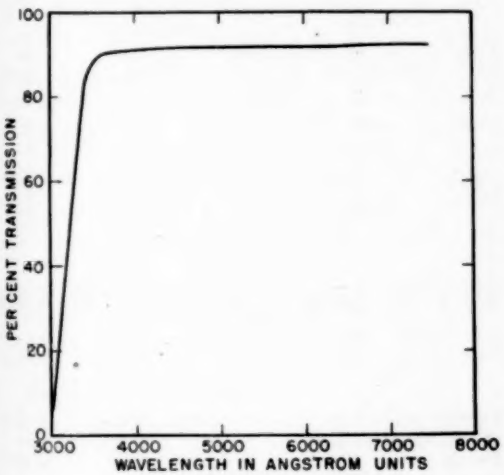


Fig. 1.—Clear glass bottle.

[1] The authors wish to express their appreciation to Mr. J. F. Greene, Director of Research, The Kimble Glass Company, for the curves on light transmission.

Clear Glass Bottles.—These were 16-oz. capacity, square, and glass stoppered. Ten grams of the powdered glass when prepared and treated in accordance with directions given in U. S. P. XIII for Type I Glass containers gave a titration of 15.8 cc. of 0.02 *N* sulfuric acid. Measurements on a sample of the glass 1.9 mm. thick averaged 91% light transmission between wave lengths 4000 Å. to 7000 Å. This curve is shown in Fig. 1.

Amber Glass Bottles.—These were square, 16-oz. capacity, and glass stoppered. Ten grams of the powdered glass when prepared and treated in accordance with directions given in U. S. P. XIII for Type I Glass containers gave a titration of 1.1 cc. of 0.02 *N* sulfuric acid. Measurements on a sample of the glass 1.8 mm. thick showed a range from 2% to 70% light transmission between wave lengths 4000 Å. to 7000 Å. This curve is shown in Fig. 2.

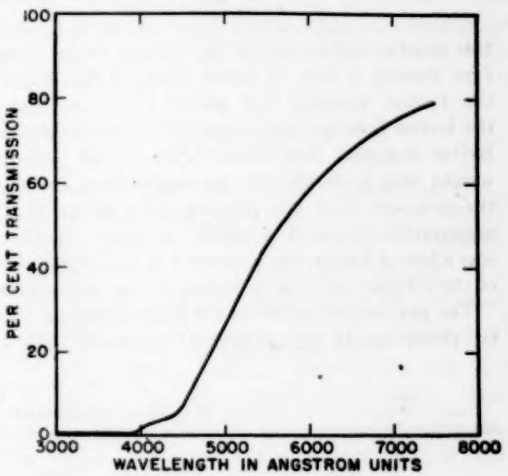


Fig. 2—Amber glass bottle.

Blue Glass Bottles.—These were round, 8-oz. capacity, and glass stoppered. Ten grams of the powdered glass when prepared and treated in accordance with directions given in U. S. P. XIII for Type I glass containers gave a titration of 13.1 cc. of 0.02 *N* acid. Measurements on a sample of the glass 2.2 mm. thick gave an irregular light transmission curve showing 88% transmission at 4000 Å., 64% at 6000 Å., and 90% at 7500 Å. This curve is shown in Fig. 3.

The bottles, filled with preparations to be tested, were placed on a shelf in the laboratory so as to duplicate usual storage conditions. These were assayed at three-month intervals and then transferred to another storage space. At no time were they protected from light in any way. For two separate three-month periods they were placed on a window sill where they were exposed to direct sunlight. One of these periods was during the summer months and on several occasions a thermometer placed near the bottles reached 40°. After

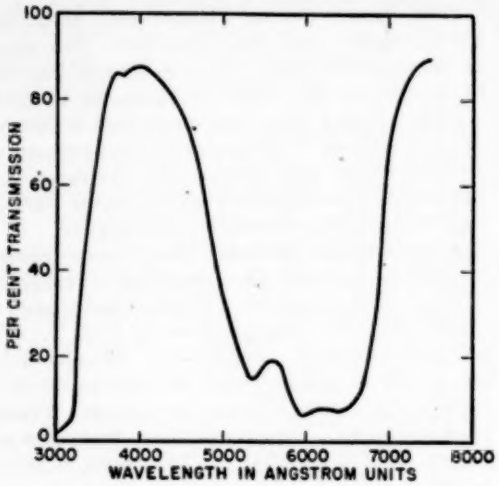


Fig. 3.—Blue glass bottle.

this drastic storage period the Strong Iodine Tincture showed a loss of about 0.5% of its weight; the Iodine Tincture lost about 0.3% in weight; the Iodine Solution lost about 0.3% and the Strong Iodine Solution lost about 0.2%. This loss in weight was probably due to evaporation through the stoppers since the percentage of iodine in all preparations showed a slight increase. If there was a loss of iodine, the loss was less rapid than that of the solvent under such drastic storage conditions.

The per cent of iodine in these preparations over the thirty-month storage period is given in Table I.

Although not recorded in the table, the iodide content of the preparations showed no significant changes. The final *p*H values were determined with a glass electrode. In such unbuffered solutions this figure may be meaningless, but it indicates that no appreciable acidity was developed. It may be observed from Table I that the only significant change in iodine content has occurred in the amber bottle containing the Iodine Solution. The iodine content of this bottle dropped from 1.93% to 1.75%, or a loss of slightly over 9% of the total iodine content. With this possible exception it is noticed that the transmission of light through the container has little effect on the stability of the preparations; the indications are that light is a minor factor influencing the stability. It is believed that the slight differences noticed are not significant in the therapeutic usefulness of iodine preparations. It may be concluded from this study that these iodine preparations when stored according to U. S. P. specifications, in tight containers with iodine resistant closure, may be expected to maintain their required strength for several years. The requirement for light-resistant containers for Strong Iodine Tincture appears to be unwarranted.

Iodine Tincture in Use in First-Aid Cabinets

Four liters of Iodine Tincture were prepared in accordance with U. S. P. XIII instructions and placed in 2-L., clear, glass-stoppered stock bottles. One of the stock bottles was stored in the dark for one year and the other was used to fill 2-oz., clear, glass-stoppered bottles which were placed in first-aid cabinets and subjected to continued use for one year. The only attention given the tinctures during the year was to remove them from the cabinets before they were entirely empty so that some sample would be available for analysis at the end of the year test period. This was necessary in the case of 2 bottles, which contained only 5 to 10 cc. when removed. Table II summarizes the results of this study.

One must conclude that Iodine Tincture is relatively stable under conditions of use. There is a

TABLE I.—STABILITY OF IODINE PREPARATIONS

	Type Bottle	Per Cent Iodine: Freshly Prepared	3 Mo. Aging	6 Mo. Storage	18 Mo. Storage	30 Mo. Storage	Final *p*H
Iodine Tincture	Clear	1.95	1.93	1.94	1.91	1.99	3.8
U. S. P. XIII	Amber	..	..	1.96	1.94	2.00	3.8
	Blue	..	..	1.95	1.91	1.99	3.9
Strong Iodine Tincture	Clear	6.94	6.86	6.92	6.90	7.04	4.7
N. F. VIII	Amber	..	..	6.92	6.88	7.06	4.7
	Blue	..	..	6.92	6.93	6.91	5.4
Iodine Solution	Clear	1.95	1.93	1.93	1.89	1.90	7.5
N. F. VIII	Amber	..	..	1.83	1.78	1.75	7.0
	Blue	..	..	1.89	1.84	1.82	7.5
Strong Iodine Solution	Clear	4.95	4.91	4.94	4.89	4.98	7.5
U. S. P. XIII	Amber	..	..	4.95	4.89	4.99	7.5

TABLE II.—IODINE TINCTURE IN FIRST-AID CABINETS

	Iodine %	NaI, %	*p*H
Freshly prepared	2.00	2.20	6.6
Stock sol. after 1 yr.	1.99	2.20	4.0
Bottle 1 after 1 yr.	2.14	2.37	3.9
Bottle 2 after 1 yr.	2.06	2.29	4.0
Bottle 3 after 1 yr.	2.01	2.26	3.9
Bottle 4 after 1 yr.	2.06	2.30	4.0
Bottle 5 after 1 yr.	2.01	2.36	4.0

slight increase in both iodide and iodine content. This is probably due to evaporation of solvent. The *p*H of the solution shows that no appreciable acidity is developed. Although the freshly prepared solution is given as *p*H 6.6, it is normal for such solutions to drop to *p*H 5 within twenty-four hours after preparation. These results are contrary to the fact that a 2% Iodine Tincture is sometimes found on the market with a low iodine content. The loss of iodine in such cases is no doubt due to the absorption by iodine reactive closures.

Studies on Old Strong Iodine Tincture Samples

Thirteen bottles of Strong Iodine Tincture were collected from individuals and analyzed. These samples have no known history other than that they were purchased by the individuals as Tincture of Iodine U. S. P. (labeled in accordance with U. S. P. XI or XII) some probably five years previous to our collection. Since no previous analysis had been made, it can only be assumed that the samples were originally within the limits set by the U. S. P. Some of the closures were faulty, and some labels showed evidence of considerable age. Results of these analyses are shown in Table III.

TABLE III.—ANALYSIS OF OLD STRONG IODINE TINCTURE SAMPLES

No.	Iodine, %	KI, %
1[a]	14.5	16.5
2	7.4	5.6
3	9.3	9.7
4	7.1	5.2
5	7.3	5.7
6	7.1	5.6
7	10.7	10.8
8	8.1	7.4
9	8.2	7.7
10	6.9	5.4
11[a]	16.3	14.8
12	7.7	5.9
13	8.6	7.2

[a] Closures in bad condition.

It may be observed that 8 of the 13 samples exceed 7.5% iodine, whereas 11 of the 13 samples exceed 5.5% potassium iodide, the maximum limits set by the official compendia. No significance can be attached to these figures other than that of actual analyses of household-used Tincture of Iodine. The samples which were found to be extremely high, e.g., Nos. 1, 7, and 11, were obviously very old and showed visible evidence of evaporation. Improperly stored samples such as these have probably given rise to the belief that aged Strong Iodine Tincture is stronger and therefore is harmful.

SUMMARY AND CONCLUSIONS

1. It has been shown that the official iodine preparations when stored in all-glass containers are stable preparations.

2. Clear, amber, and blue bottles are equally satisfactory for storage, showing that light alone has little effect on the stability of the preparations.

3. Iodine Tincture (Mild Tincture of Iodine U. S. P. XII) in proper containers is not subject to appreciable change under actual conditions of use for a period of a year.

4. A survey of household samples of Strong Iodine Tincture has shown that these samples have a tendency to be somewhat stronger than official specifications. This tendency is probably due to improper closures.

REFERENCES

(1) "United States Pharmacopœia," 1830 edition, John Grigg, Philadelphia, Pa., 1831.
(2) *Ibid.*, S. Converse, New York, 1830.
(3) *Ibid.*, 1840 edition, Grigg and Elliott, Philadelphia, Pa., 1842.
(4) *Ibid.*, Thirteenth revision, Mack Printing Co., Easton, Pa., 1947.
(5) "National Formulary," Eighth edition, Mack Printing Co., Easton, Pa., 1946.
(6) "United States Pharmacopœia," 1850 edition, J. B Lippincott and Co., Philadelphia, Pa., 1851.
(7) *Ibid.*, Eighth revision, P. Blakison's Son and Co., Philadelphia, Pa., 1905.
(8) *Ibid.*, Ninth revision, P. Blakison's Son and Co., 1916.
(9) *Ibid.*, Eleventh revision, Mack Printing Co., 1935.
(10) La Wall, C. H., and Tice, L. F., THIS JOURNAL, **21,** 122(1932).
(11) Gershenfeld, L., and Miller, R. E., *ibid.*, **21,** 895 (1932).
(12) Karns, G. M., Cretcher, L. H., and Beal, G. D., *ibid.*, **21,** 783(1932).
(13) Biskind, M. S., *Proc. Soc. Exptl. Biol. Med.*, **30,** 35 (1932).
(14) Gershenfeld, L., and Patterson, G. W., Jr., *Am. J. Pharm.*, **117,** 5(1945).
(15) Secor, W. L., *Am. J. Surg.*, **26,** 522(1934).
(16) "United States Pharmacopœia," Twelfth revision, Mack Printing Co., Easton, Pa., 1942.
(17) Droste, R., *Pharm. Ztg.*, **57,** 166; through *Chem. Abst.*, **7,** 215(1913).
(18) Carter, R. M., *Ind. Eng. Chem.*, **18,** 827(1926).
(19) Zeuthen, H. R., *Süddeut. Apoth. Ztg.*, **75,** 512(1935).
(20) Gianturco, E. (Trans. by Biello, J. A.), *U. S. Naval Med. Bull.*, **10,** 314(1916).
(21) Bohrisch, P., *Apoth. Ztg.*, **43,** 436(1928); through *Chem. Abst.*, **22,** 2028(1928).
(22) Budde, Th., *Apoth. Ztg.*, **27,** 203(1912); through *Chem. Abst.*, **6,** 1493(1912).
(23) Johannessen, *Farmac. Tidende*, p. 46 (1912); through *Chem. Abst.*, **7,** 4043(1913).
(24) Knoll, W., *Apoth. Ztg.*, **43,** 1324(1928).
(25) Ostling, G. J., *Dansk. Tids. Farm.*, **1,** 139(1927); through *Chem. Abst.*, **21,** 2049(1927).

A Modified Kober Method for the Determination of Alpha- and Beta-Estradiol*

By JONAS CAROL and J. C. MOLITOR

A method is described for the determination of α- and β-estradiol by a modified Kober procedure. It is based on the fact that β-estradiol reacts with Kober's reagent in the cold while α-estradiol does not. The results of analysis of a series of samples containing the two isomers are given.

EXISTING chemical methods for the determination of estradiol are of limited value in estimating biological potency since they do not indicate the relative amounts of the α- and β-isomers present. Doisy, Thayer, and Van Bruggen (1) and Pearlman and Pearlman (2) have developed differential bioassays for α- and β-estradiol but these methods require too much time for general analytical use, and are subject to occasional serious error. Whitman, Wintersteiner, and Schwenk (3) have separated the α- and β-estradiols by precipitating the insoluble α-estradiol digitonide. This method was not applicable to samples containing microgram quantities of estradiols. Attempts to separate the isomers by absorption chromatographic methods similar to those used by Stimmel (4) for the separation of estrone, estradiol, and estriol failed as did attempts to make the separation by partition chromatography.

The method of analysis presented in this paper is based on the fact, not previously reported, that in the initial step of the Kober reaction (5) β-estradiol reacts with the reagent at room temperature, while α-estradiol[1] does not. Under these conditions completion of the reaction produces the normal magenta colored solution with the β-isomer and a colorless solution with the α-isomer. By use of this modified procedure, α-estradiol is determined as the difference between total estradiols and β-estradiol. For simplicity of presentation determinations made with the initial reaction carried out at the temperature of boiling water will hereafter be referred to as procedure *A*, and those made with the initial reaction at room temperature will be referred to as procedure *B*. In both procedures, the remainder of the reaction is carried out essentially as directed by Venning, *et al.* (6).

EXPERIMENTAL

Apparatus.—A spectrophotometer or photometer equipped to measure extinction at 420, 526, and 528 mμ. (A Beckman Quartz Spectrophotometer fitted with matched 1-cm. absorption cells was used in this investigation.)

Reagents.—α-Estradiol, m. p. 175–176°, standard solution prepared in 95% alcohol containing 100 micrograms per ml.

β-Estradiol, m. p. 220–222°, standard solution prepared in 95% alcohol containing 50 micrograms per ml.

Kober's Reagent—Slowly add, with mixing, 5.6 parts (wt.) of C. P. grade sulfuric acid to 3.6 parts (wt.) of freshly redistilled phenol. Cool to prevent temperature rise.

Dilute sulfuric acid 25% *v/v*.

Determination of the optimum time for completion of the initial phase of the reaction of β-estradiol in procedure *B* was necessary as a preliminary to the remainder of the investigation. In a series of determinations made with 50-microgram samples of β-estradiol, the time of the initial phase of the reaction was increased by fifteen-minute intervals. The extinction values of the resultant solutions, measured at 523 mμ, and shown in Table I, indicate that the initial reaction is completed within fifteen minutes. Further increases in reaction time result in a gradual loss in color. In the following work an initial reaction period of twenty minutes was used in procedure *B*, so as to conform with that specified by Venning, *et al.* (6), and used in procedure *A*.

The absorption spectra of the solutions formed in procedure *A* with 100-microgram samples of α- and β-estradiol, and the absorption spectrum of the solutions formed in procedure *B* with β-estradiol, were measured from 420–580 mμ. The curves shown in Fig. 1 show that while the absorption spectrum of the color formed by the α-isomer is essentially the same as reported by Venning, *et al.* (6), with a maximum at 523 mμ, the absorption spectra of the

* Received Jan. 31, 1947, from the Medical Division, Chemical Section, Food and Drug Administration, Federal Security Agency, Washington, D. C.

[1] Estrone, estriol, and equilenin, like α-estradiol, do not react with Kober's reagent at room temperature. A very small sample of equilin obtained by the writers produced a bright yellow color in both the initial and final phases of the reactions. No further work has been done to determine if this is a typical color or if it is formed by an impurity. We are indebted to Dr. Oliver Kamm, of Parke, Davis & Company, who supplied us with the samples of equilin and equilenin.

TABLE I.—VARIATION OF EXTINCTION VALUE WITH TIME OF INITIAL REACTION OF SOLUTIONS PREPARED FROM β-ESTRADIOL BY PROCEDURE *B*

Time in Min.	$E_{528m\mu}$, 1 cm.[a]
15	0.323
30	0.314
45	0.295
60	0.285
75	0.272
90	0.263
105	0.259
120	0.248

[a] These measurements were made before it was found that the maximum for the solution formed with β-estradiol is at 528 mμ.

color formed by the β-isomer in both procedures *A* and *B* have maxima at 528 mμ; and that the extinction coefficient at 528 mμ of the product formed with β-estradiol by procedure *B* is about 60% greater than that formed by procedure *A*.

The validity of Beer's law when applied to spectrophotometric measurements made on products formed by β-estradiol in procedure *B*, with and without added α-estradiol, was determined. Two series of solutions were prepared, one from 10–50-microgram samples of β-estradiol, and the other from 10–50-microgram samples of β-estradiol each containing 100 micrograms of added α-estradiol. Extinction measurements were made on these solutions at 528 mμ and these data are recorded in Table II.

A plot of these extinction values against concentration of β-estradiol, as shown in Fig. 2, establishes adherence to Beer's law and indicates no interference from added α-estradiol.

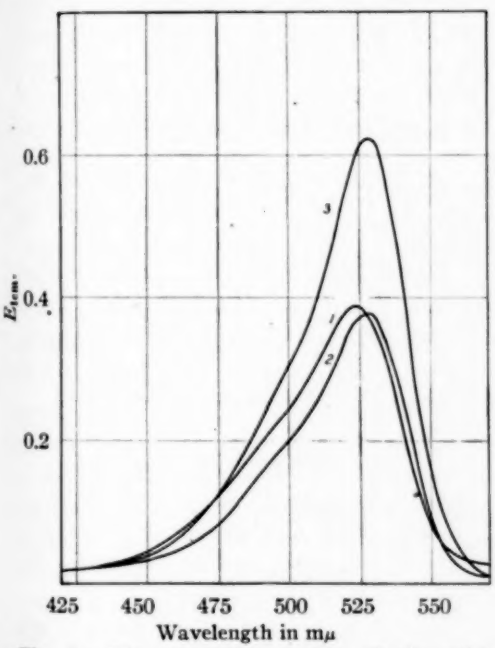


Fig. 1.—The absorption spectra of the colors formed from 100 microgram samples of α- (curve 1) and β- (curve 2) estradiol by procedure a, and β-estradiol (curve 3) by procedure b.

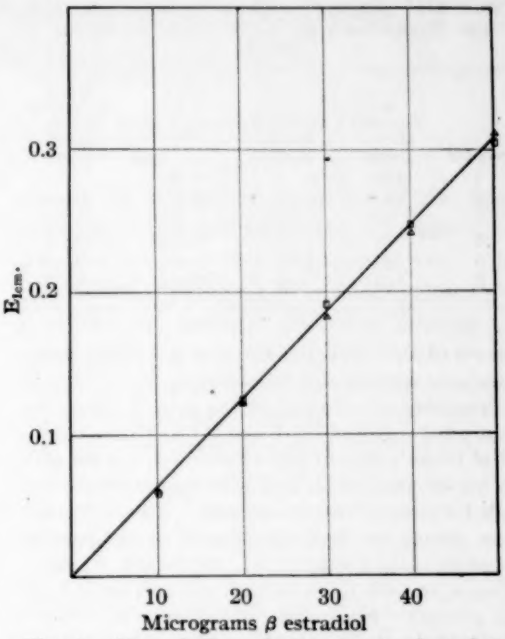


Fig. 2.—Plot of E against concentration of β-estradiol at 528 mμ. Δ without added α-estradiol. □ with 100 micrograms added α-estradiol.

TABLE II.—APPLICABILITY OF BEER'S LAW TO PRODUCTS FORMED BY β-ESTRADIOL IN PROCEDURE *B*

β-Estradiol, Micrograms	$E_{1\ cm.\ 528m\mu}$ Without Added α-Estradiol	With Added α-Estradiol
10	0.059	0.053
20	0.118	0.121
30	0.179	0.190
40	0.237	0.246
50	0.310	0.302

It is evident from a study of the absorption data in Fig. 1 that extinction measurements made at the maximum for the α-estradiol color may not give correct results for total estradiols when an appreciable amount of β-estradiol is present. Determinations of total estradiols made from extinction measurements at 526 mμ, the region of equal extinction for both the α- and β-estradiol color (by procedure *A*) should give correct results regardless of the proportion of α- and β-estradiol in the sample. A series of solutions was prepared, by procedure *A*, from mixtures of estradiols varying from 100% of α to 100% of β. Extinction readings of these solutions were made at 526 mμ and the amounts of total estradiols were calculated using α-estradiol as a standard. These results are presented in Table III.

Method.—To each of two 20 × 150 mm. test tubes transfer aliquots of an alcoholic solution of the sample containing about 25–100 micrograms of total estradiols. Transfer 1 ml. of α-estradiol standard solution to a third test tube, and 1 ml. of β-estradiol standard solution to a fourth tube. Evaporate each to dryness on a steam bath with aid of a

TABLE III.—RESULTS OF THE DETERMINATION OF TOTAL ESTRADIOLS BY EXTINCTION READINGS AT 526 mμ

Sample	α-Estradiol Micrograms	β-Estradiol Micrograms	$E_{1\text{ cm. }526 m\mu}$	Total Estradiols Found, Micrograms
1	100	0	0.369	...
2	80	20	0.363	98.4
3	60	40	0.364	98.6
4	40	60	0.369	100.0
5	20	80	0.364	98.6
6	0	100	0.352	95.7

current of air. Dry the tubes in a vacuum desiccator over sulfuric acid for one hour.

Procedure *A*.—To one of the sample tubes, the α-estradiol standard tube, and a blank tube, add 3 ml. of Kober's reagent (from a burette), stopper with tin foil covered corks, and heat in a boiling-water bath for exactly twenty minutes. Mix each tube twice during the first ten minutes of the heating period by shaking vigorously. At the end of twenty minutes, transfer to an ice bath and cool for at least five minutes. Allow tubes to remain in ice while 3 ml. distilled water is added from a burette to each test tube and the contents are thoroughly mixed with a glass rod. Leave the rods in the test tubes for the remainder of the reaction. Place the tubes again in the boiling water bath and heat for exactly three minutes. At the end of three minutes transfer the tubes again to the ice bath and cool for at least five minutes. After cooling, transfer the contents of each tube to a glass-stoppered graduated cylinder and dilute to 15 ml. with dilute sulfuric acid.[2] Mix well and determine the extinction of both the sample solution and α-estradiol standard relative to the blank solution at 420 mμ[3] and 526 mμ.

Micrograms total estradiols in aliquot =

$$\frac{E_{526\ m\mu\ sample} - \frac{E_{420\ m\mu\ sample}}{2}}{E_{526\ m\mu\ standard} - \frac{E_{420\ m\mu\ standard}}{2}} \times 100$$

Procedure *B*.—To the other sample tube, the β-estradiol standard tube, and a blank tube, add from a burette 3 ml. Kober's reagent and mix each tube thoroughly with a glass rod. Leave the rods in the tubes for the remainder of the reaction. Allow the tubes to stand at room temperature for twenty minutes, stirring every five minutes. At the end of twenty minutes, transfer to an ice bath and cool for at least five minutes. Allow tubes to remain in ice while 3 ml. distilled water is added from a burette to each test tube and the contents are thoroughly mixed with a glass rod. Place the tubes in a boiling-water bath and heat for exactly three minutes. At the end of three minutes transfer the tubes again to the ice bath and cool for at least five minutes. After cooling, transfer the contents of each tube to a glass-stoppered graduated cylinder and dilute to 15 ml. with dilute sulfuric acid. Mix well and determine the extinction of both the sample solution and the β-estradiol standard relative to the blank solution at 420 mμ and 528 mμ.

Micrograms β-estradiol in aliquot =

$$\frac{E_{528\ m\mu\ sample} - \frac{E_{420\ m\mu\ sample}}{2}}{E_{528\ m\mu\ standard} - \frac{E_{420 m\mu\ standard}}{2}} \times 50$$

α-estradiol = total estradiols − β-estradiol

A series of mixtures of α-and β-estradiol was prepared and analyzed by the above method. The results of analysis are shown in Table IV.

TABLE IV.—RESULTS OF ANALYSIS OF KNOWN MIXTURES OF α-AND β-ESTRADIOL

Sample	α-Estradiol Micrograms Added	α-Estradiol Micrograms Found	β-Estradiol Micrograms Added	β-Estradiol Micrograms Found
1	5	4.2	100	99.5
2	10	9.0	90	88.1
3	20	24.9	20	20.1
4	20	21.5	80	76.6
5	30	33.4	30	30.4
6	40	41.0	25	27.0
7	40	43.6	40	38.4
8	50	44.0	50	50.2
9	60	57.9	40	40.1
10	75	70.0	35	38.0
11	80	79.2	20	19.7

SUMMARY AND CONCLUSIONS

1. A modified Kober procedure is described for the determination of α- and β-estradiol.

2. Results of analysis of a series of mixtures of α- and β-estradiol are given. Good results are obtained with mixtures containing as little as 5 micrograms of one isomer in the presence of 100 micrograms of the other.

REFERENCES

(1) Doisy, E. A., Thayer, S. A., and Van Bruggen, J. T., *Federation Proc.*, **1**, 202(1942).
(2) Pearlman, W. H., and Pearlman, M. R. J., *Arch. Biochem.*, **4**, 97(1944).
(3) Whitman, B., Wintersteiner, O., and Schwenk, E., *J. Biol. Chem.*, **118**, 789(1937).
(4) Stimmel, B. F., *ibid.*, **162**, 99(1946).
(5) Kober, S., *Biochem. Z.*, **239**, 209(1931).
(6) Venning, E. H., Evelyn, K. A., Harkness, E. V., and Browne, J. S. L., *J. Biol. Chem.*, **120**, 225(1937).
(7) Bachman, C., and Pettit, D. S., *ibid.*, **138**, 689(1941).

[2] The color of solutions prepared by the writers according to Venning, *et al.* (6), faded too rapidly to permit accurate colorimetric measurements. Increasing the concentration of the sulfuric acid used to dilute the reaction mixture from 10% to 25% v/v produced colors that remained stable at room temperature.

[3] This correction is based on the findings of Venning, *et al.* (6), Bachman and Pettit (7), and Stimmel (4) that $E_{528 m\mu}/E_{420 m\mu}$ for nonestrogenic impurities is approximately 0.5.

Availability of Penicillin from Various Ointment Bases*

By H. A. CLYMER and R. J. FERLAUTO

The release of penicillin from various type ointment bases by methods which are believed to simulate closely actual *in vivo* conditions has been investigated. Data from the methods employed give actual concentration figures of the amount of penicillin released by the various type bases. Results obtained are proposed as the basis for a more rational means of choosing bases for penicillin to be used topically.

DURING the past ten years the term *topical application of ointments* has broadened in meaning both as to actual therapeutic intent and as to the type of bases employed. For centuries this implied empirical application by inunction of medicaments contained in fatty bases and was concerned primarily with the treatment of superficial skin disorders or with the attempt to obtain systemic absorption by penetration. With the advent of the newer, more specific, and less toxic antibacterial agents, the practice has become accepted of applying these substances directly to the tissues as anti-infective agents in the treatment of wounds, burns, and in minor surgery. Thus can be achieved the objectives of topical application in attaining high tissue concentrations of the drug at the site of the infection in shorter periods of time and with less drug than by systemic administration. While this change in therapeutic intent has been taking place, advancement has also been made in the bases to be used as vehicles. This has been primarily due to the development of synthetic emulsifying agents, making possible easy preparation of a wide variety of water miscible oil-in-water emulsions. Chamings (1) in a recent address traces this same development of ointment medication from the days of "empiricism" through the present state of experimentation and stresses that the ultimate aim of efficient ointment therapy will only be gained by intelligent choosing of the base from the types now available after thorough consideration of the therapeutic end. In view of these changes, it is no longer simply a matter of placing the new therapeutic agent into Simple Ointment U. S. P. in order to formulate the topical form required. This last statement should be self-evident, but the incidence of this type of formulation of topical therapy has been so frequent the authors feel reiteration to be mandatory.

There has been considerable effort on the part of the pharmaceutical and medical professions to set up specifications for vehicles and bases for topical application. These listings cover requirements ranging from chemical inertness to the cosmetic niceties. They serve as excellent check lists for the compounder when contemplating topical medication. One must not lose sight, however, of the fact that the primary and indispensable quality of a carrier is that it allows adequate release of the active therapeutic medication. The lack of this quality nullifies whatever other value the base may possess. It has been pointed out (2, 3) that many apparent therapeutic failures with sulfonamides were due to improper topical application of the drug with resulting low local tissue concentrations of the drug. In order to avoid a repetition of this type of failure, it is doubly important with the topical use of penicillin that adequate concentrations are maintained, for Molitor (4) has warned that insufficient tissue concentrations create the possibility of pathogenic organisms' acquiring resistance to penicillin. This problem of acquired drug resistance of pathogenic organisms makes the use of preparations affording inadequate release of penicillin not just wastefully ineffective but potentially dangerous.

The present status of bases for penicillin ointment is unsettled. In this country penicillin ointment intended for sale in interstate commerce is limited by the F.D.A.

* Received Nov. 25, 1946, from the Research Laboratories, Smith, Kline, and French Laboratories, Philadelphia, Pa.
Presented to the Scientific Section, A. PH. A., Pittsburgh meeting, 1946.

Regulation Sec. 146.26 (5) to all grease bases composed primarily of wool fat, petrolatum, or white petrolatum, or any mixture of two or all of these, the use of certain other fats and waxes being optional. The new Addendum (6) to the British Pharmacopœia includes official monographs for an all grease preparation and two oil-in-water emulsion creams. The final choice of bases for topical application of penicillin, as with other medicinal agents, will have to come from elaborate, controlled clinical investigations; reports of such investigations, however, are meager and inconclusive. It is important for sound clinical evaluation to possess a preliminary idea, obtained from adequately controlled *in vitro* tests, of the inherent properties of the medicinal substances when incorporated into the various bases. The lack of this type of information prompted the present investigation.

EXPERIMENTAL PLAN

In any *in vitro* evaluation of antiseptics the basic objective must be to simulate as closely as possible the conditions under which the preparation is to be used *in vivo*. We have limited our testing to that phase of topical application whereby anti-infective agents are applied directly to the tissue, as in the treatment of wounds and burns, and in minor surgery and have not been concerned with application by inunction to intact skin. The following factors were, therefore, considered in choosing the methods to be used:

(*a*) epidermal layer of the skin has been removed, exposing serous tissue fluid; and

(*b*) penicillin concentrations once achieved are not static since tissue fluids are constantly changing.

The standard agar diffusion method (7) for testing antiseptic ointments was considered grossly inadequate. Even though appreciable zones of inhibition are obtained with both water-miscible and water-immiscible bases by this method the results indicate only that a sufficient amount of the drug has been made available to produce the inhibition zone under the conditions of the test. Information cannot be obtained as to the quantity or continuity of release of the drug from the base. The method employed by Waud and Ramsay (8) and by Huyck, *et al.* (9) to determine the availability of sulfonamides in topical form is a modification of the agar diffusion test employing chemical indicator rather than bacterial inhibition. The method of Bandelin and Kemp (3) is deemed superior in that it gives some idea of the quantitative release of the drug, but it allows for a variance in surface area and does not simulate change of tissue fluid.

Simulation of *in vivo* conditions to obtain data on the continuity and quantity of release of penicillin was thought to be met best by the following method: Ointments were enclosed in cellophane bags to facilitate repeated transfer of the ointment bulk from first diffusion fluid to successive, fresh fluids and determining the amount of penicillin released in each diffusate. Information as to the continuity of release was adequately obtained by this method. When data so obtained were interpreted as to accurate quantitative release, however, the following shortcomings were observed: rigid control of surface area was impossible, the volume of diffusion media needed was quite large, and the cellophane bag set up a possible barrier which is not present *in vivo*. An improved method was developed whereby the ointment was contained in a shallow glass cup, affording a uniform surface area and allowing a minimum of diffusion media. Serum was substituted for saline, since it more closely simulates tissue fluid. The practice of transferring the ointment from the first diffusion fluid to successive, fresh fluids and determining the amount of penicillin released in each diffusate was again employed.

In choosing the bases to be used in this evaluation, we have confined ourselves mainly to the all grease bases as representative of water-immiscible vehicles, and to oil-in-water emulsions as representatives of water-miscible bases. Water-in-oil emulsions were not used extensively since from preliminary experimentation the release was found to be of the same magnitude as from all grease. The water-soluble base, Carbowax, although showing excellent liberation, was deemed impractical because of the relative instability of penicillin in this compound.

EXPERIMENTAL

The petrolatum,[1] petrolatum-plus-lanolin,[2] and oil-in-water emulsion[3] bases were prepared and then autoclaved. Upon cooling, sufficient calcium penicillin[4] was incorporated so that the finished ointment would contain approximately 1000 u./Gm. An original assay for actual penicillin content was performed on each ointment immediately prior to use.

Continuity of Release (Cellophane Bags).—Visking tubing with an inflated diameter of 1.9 cm. was cut in lengths of approximately 10 cm. One end was tied and then placed in boiling water for three minutes. After draining, 4 Gm. of the ointment to be used was placed into each bag and sealed. The bags were suspended in test tubes, 2.5 cm. x 20 cm., containing 15 cc. of sterile normal saline solution. The bags were weighted so that they remained completely immersed in the saline. The test tubes were closed with rubber stoppers and placed at 37° in an incubator on a rotary agitator so that a gentle swirling action was obtained. After the diffusion

[1] White Perfecta, Sonneborn.
[2] 10% Anhydrous Lanolin, U. S. P.; 90% Petrolatum, U. S. P. (White Perfecta, Sonneborn).
[3] Sodium Lauryl Sulfate 1%. Cetyl Alcohol 10%. Mineral Oil 20%. Glycerin 10%. Distilled Water 59%.
[4] Pfizer, Lot 1955C.

period, the bags containing the ointment were lifted out of the saline solution and allowed to drain in the tube. They were then immediately transferred and immersed in a new tube containing a fresh 15-cc. portion of normal saline for the next diffusion period. This procedure was carried out for four successive diffusion intervals of 3, 3, 16, and 8 hours respectively. The tubes of saline after the diffusion periods were stored under refrigeration until they were assayed for penicillin content.

Constant Surface Area (Shallow Cup).—Each of the samples of ointment to be tested was placed in a containing glass cup with an internal diameter of 3.5 cm. and an internal depth of 0.3 cm. These cups were specially constructed to insure uniform diameter and provided a constant surface area of 9.6 sq. cm. Since the surface area of each of the ointments was to be the same, great care was taken to obtain a smooth continuous level surface of the ointment in the cup. The cup containing the ointment was then placed in a porcelain dish of slightly larger dimensions, i.e., diameter 4.5 cm. and depth of 1.2 cm. Seven cubic centimeters of human serum[5] were added to cover the ointment surface completely, taking care not to disturb the surface of the ointment. The cups were then placed at 37° in an incubator and covered to minimize evaporation. At the end of the diffusion period the inner cup containing the ointment was carefully removed and serum allowed to drain into the outer cup. The cups were gently passed through sterile distilled water to remove adhering serum and then transferred to new outer cups and 7 cc. of serum added. This procedure was repeated for four successive diffusion periods of 8, 2, 2 and 2 hours respectively. The serum diffusates were refrigerated (5°) until assayed for penicillin content.

The assays for penicillin content of the ointments and diffusates were carried out as follows: petrolatum-plus-lanolin ointments were assayed by the F.D.A. agar diffusion method (10) for the determination of potency of penicillin ointments. Since an oil-in-water emulsion is water miscible, it was uniformly dispersed with mechanical agitation in phosphate buffer (*p*H 6) and then assayed by the standard F.D.A. method for penicillin solutions. Assays for penicillin content of the diffusates were carried out employing the standard F.D.A. agar diffusion method for penicillin solutions. Where penicillin concentrations were high the diffusates were diluted to approximately 1 u./cc. of penicillin with phosphate buffer (*p*H 6).

RESULTS

Results from the principal experiments described above are summarized in Tables I and II.

Control tests were performed in the following manner. First, the rates of inactivation of penicillin in serum and saline under the conditions of the test were determined and are recorded as the controls in Tables I and II. The rate of inactivation of penicillin in the serum after eight hours appears exces-

TABLE I.—CONTINUITY OF RELEASE OF PENICILLIN FROM TWO OINTMENT BASES CONTAINED IN CELLOPHANE BAGS[a]

		Ointment Potency, u./Gm.	Diffusion Periods I 3 Hr., u./cc.	II 3 Hr., u./cc.	III 16 Hr., u./cc.	IV 8 Hr., u./cc.
Petrolatum	#1	895	0.2	0.14	0	0
	#2	895	0.27	0.13	0	0
O/W Emulsion	#1	1260	52.0	19.8	37.5	9.0
	#2	1260	56.0	18.6	35.0	10.3

[a] Control—Calcium penicillin in normal saline lost approx. 8–9% in 16 hr. at 37°.

TABLE II.—COMPARATIVE RELEASE OF PENICILLIN FROM VARIOUS OINTMENT BASES EMPLOYING THE SAME SURFACE AREA[a]

		Ointment Potency, u./Gm.	Diffusion Periods I 8 Hr., u./cc.	II 2 Hr., u./cc.	III 2 Hr., u./cc.	IV 2 Hr., u./cc.
Petrolatum	#1	820	0.184	0	0	0
	#2	820	0.155	0	0	0
Petrolatum Lanolin	#1	810	0.163	0	0	0
	#2	810	0.175	0	0	0
O/W Emulsion	#1	890	75.5	17.8	10.8	9.4
	#2	890	73.5	14.6	11.4	11.3

[a] Control—Stability of calcium penicillin in human serum[b]

Original Potency (50 u/cc.)	% Activity Remaining After 2 hr.	8 hr.
100%	92	40

[b] The serum for this experiment was prepared aseptically by collecting blood directly from the vein, into a bottle containing mineral oil. It was then centrifuged under paraffin and the serum withdrawn and kept layered with mineral oil until used.

sive; serum *p*H, however, had risen to 8.8 by the end of the diffusion period. Serum containing penicillin with a protective layer of mineral oil to prevent carbon dioxide loss remained practically unchanged in *p*H and the rate of inactivation of penicillin was considerably less. In the second control measure, each ointment base was placed in serum and saline containing penicillin under the conditions of the test; the presence of these bases caused no increase in the rate of penicillin inactivation.

DISCUSSION

Analysis of the diffusates after contact with the ointment shows that penicillin in an all-grease base is relatively unavailable to serum or saline. There was a small immediate release but none thereafter, indicating that only the penicillin on the surface of these ointments was available and the bulk of the penicillin remained locked in the base. Release of penicillin from the oil-in-water emulsion bases, however, was high in the first diffusion period, leveling off to a constant high concentration throughout the entire period of testing. The physical nature of the oil-in-water emulsion base undoubtedly is responsible for this effect since the continuous phase of the emulsion is miscible with the surrounding fluid media and allows diffusion to take place. These results parallel what has been found with the sulfonamides.

In order to determine from the standpoint of penicillin stability the feasibility of using the oil-in-water emulsion base, the stability under refrigerated conditions, i.e., 5°, was tested and the results show that 80 per cent of the original penicillin content is available after one month's storage and 50 per cent after two months' storage. This indicates that oil-in-water emulsion is satisfactory for use in compounding extemporaneous penicillin ointments. It must be stressed, however, that the stability is for only the oil-in-water emulsion base used in this experiment, and that any modification would necessitate rechecking to determine the effect upon stability of the penicillin.

SUMMARY

Two *in vitro* methods for the estimation of the amount and continuity of release of penicillin in various ointment bases are described. By these methods an evaluation of penicillin availability from representative water-miscible and water-immiscible bases has been obtained. The quantity of penicillin released was much greater with the oil-in-water emulsion than with the all-grease base. No continuity of release was found with the all-grease base from which there was only a small immediate release and none thereafter. Water miscible oil-in-water emulsion base, however, showed a high continuous release. It is concluded that the use of water-miscible oil-in-water emulsion bases are superior in making penicillin available to tissue fluids.

REFERENCES

(1) Chamings, A. R. G., *Pharm. J.*, **156,** 269(1946).
(2) Editorial, *U. S. Naval Med. Bull.*, **44,** 861(1945).
(3) Bandelin, F. J., and Kemp, C. R., THIS JOURNAL, **35,** 65(1946).
(4) Molitor, H., *Federation Proc.*, **5,** 304(1946).
(5) *Fed. Reg.*, Sept. 1, 1945, p. 11233.
(6) *Pharm. J.*, **156,** 345(1946).
(7) F.D.A., "Methods of Testing Antiseptics & Disinfectants," Circular 198, p. 12.
(8) Waud, R. A., and Ramsay, A., *Can. Med. Assoc. J.*, **48,** 121(1943).
(9) Huyck, C. E., Hirose, R. S., and Reyes, P. A., THIS JOURNAL, **35,** 129(1946).
(10) *Fed. Reg.*, Sept. 8, 1945, p. 11478.

Some Minor Alkaloids of Pei-Mu, Fritillaria Roylei*

By T. Q. CHOU†

Four minor alkaloids in addition to the two principal ones previously reported have been isolated from the Chinese drug, Pei-Mu. The procedures employed are described and the analytical data and derivatives used in the characterization of these new alkaloids are reported.

IN A PREVIOUS communication, Chou and Chu (1) reported the preparation and properties of peimine, $C_{26}H_{43}O_3N$ and peiminine, $C_{26}H_{41}O_3N$, the two principal alkaloids of the Chinese drug, Pei-Mu, identified as *Fritillaria Roylei*. From their mother liquor, there have been isolated up to present 4 other alkaloids which are named peimisine, peimiphine, peimidine, and peimitidine, respectively. Their compositions, melting points, and specific rotations are given in Table I.

TABLE I

Name	Formula	M. P., °C.	Specific Rotation
Peimisine	$C_{27}H_{43}O_4N$	270	$[\alpha]_D^{25}$ −51° in alcohol
Peimiphine	$C_{27}H_{45}O_3N$	127	$[\alpha]_D^{31}$ −69° in alcohol
Peimidine	$C_{27}H_{43}O_2N$	222	$[\alpha]_D^{25}$ −74° in alcohol
Peimitidine	$C_{27}H_{41}O_3N$	188	$[\alpha]_D^{30}$ −68° in alcohol

All these alkaloids are found in the drug only in very small quantities, ranging from 0.001 to 0.002 per cent. Their isolation has been effected by taking advantage of the difference in the solubilities of their well-crystallized hydrobromides or hydrochlorides. Like peiminine, peimisine contains in its molecule a carbonyl group, forming easily an oxime, m. p. 196°.

EXPERIMENTAL

Peimisine, $C_{27}H_{43}O_4N$.—In the isolation of peimine and peiminine as reported previously (1), 70 Kg. of the Chinese drug, Pei-Mu, were used. After the removal of these 2 principal alkaloids in the form of their hydrochloride as much as possible by fractional crystallization, all the mother liquors are united and distilled nearly to dryness and the residue taken up with water in which much resinous matter remains insoluble. The aqueous solution is filtered, made alkaline with sodium carbonate, and the precipitate extracted with ether. The ethereal solution, when dried and distilled, leaves behind about 10 Gm. of a basic residue which is dissolved in acetone and neutralized with an alcoholic hydrochloric acid. On standing for a few weeks at room temperature, there separates out a crystalline deposit weighing about 1.5 Gm. and consisting of a mixture of peimisine, peimiphine, and peiminine hydrochlorides. After filtration, it is warmed on water bath for some time with 10 cc. of 93% alcohol in which about half the quantity remains insoluble The insoluble part which consists of the peimisine hydrochloride in an almost pure state is filtered and dried, its mother liquor being reserved for working up peimiphine. It is dissolved in about 70 cc. of boiling water. The aqueous solution, when cooled to room temperature, is made alkaline with sodium carbonate and the liberated base extracted with chloroform. Peimisine is recovered from the chloroform solution by distilling off the solvent and crystallizing the residue from a little alcohol. It forms colorless rhombic prisms, m. p. 270°, $[\alpha]_D^{25}$ −51. It is easily soluble in alcohol and chloroform, but only sparingly soluble in acetone or ether. Its hydrochloride, prepared by neutralizing the pure alkaloid with hydrochloric acid in alcohol, forms prismatic needles, m. p. 257°, and is soluble in water or alcohol with difficulty. Its aurichloride is obtained as an amorphous powder by precipitation in aqueous solution in the presence of hydrochloric acid. The analytical data obtained with the alkaloid, its hydrochloride, and aurichloride agree with the formula $C_{27}H_{43}O_4N$ as shown in Table II.

Peimisine Oxime, $C_{27}H_{44}O_4N_2$.—A mixture of 0.1 Gm. of peimisine, 0.1 Gm. of hydroxylamine hydrochloride, and 0.2 Gm. of potassium acetate is dissolved in 20 cc. of water in the presence of one drop of acetic acid and warmed on the water bath for about one hour. After standing overnight the clear aqueous solution is made alkaline with potassium carbonate and the precipitate extracted with a mixture of ether and chloroform. The ether-chloroform extract, when dried and distilled, leaves behind the required oxime which separates from acetone as a crystalline powder, m. p. 196°. Its hydrochloride crystallized from alcohol in prismatic

* Received Dec. 30, 1946, from the Institut de Materia Medica, National Academy of Peiping, Shanghai, China.
† Acknowledgment is made to Dr. T. T. Chu of this Institute for carrying out all the micro-analyses indicated in this paper.

TABLE II.—CHEMICAL COMPOSITION OF THE COMPOUNDS DESCRIBED IN THIS PAPER

Name	Formula	Percentage Composition											
		Carbon		Hydrogen		Nitrogen		Chlorine		Platinum or Gold	
		Calcd.	Found	Calcd.	Found	Calcd.	Found	Calcd.	Found	Calcd.	Found
Peimisine	$C_{27}H_{43}O_4N$	72.75	72.78	9.73	9.81	3.14	3.26	3.09			
Peimisine Hydrochloride	$C_{27}H_{43}O_4N \cdot HCl$							7.35	7.43	7.58	
Peimisine Aurichloride	$C_{27}H_{43}O_4N \cdot HCl \cdot AuCl_3$									25.10	24.93
Peimisine Oxime	$C_{27}H_{44}O_4N_2$					6.08	6.07	6.20			
Peimisine Oxime Hydrochloride	$C_{27}H_{44}O_4N_2 \cdot HCl$							7.13	7.18	7.21	
Peimiphine	$C_{27}H_{46}O_3N$	74.93	74.73	10.72	9.99	3.23	3.36	3.17			
Peimiphine Hydrochloride	$C_{27}H_{46}O_3N \cdot HCl$							7.56	7.75	7.63	
Peimiphine Aurichloride	$C_{27}H_{46}O_3N \cdot HCl \cdot AuCl_3$									25.53	25.53
Peimidine	$C_{27}H_{45}O_2N$	78.00	77.93	78.01	10.92	11.24	11.37	3.37	3.20	3.07	
Peimidine Hydrochloride	$C_{27}H_{45}O_2N \cdot HCl$							7.85	7.85	7.61	
Peimidine Platinichloride	$(C_{27}H_{45}O_2N \cdot HCl)_2PtCl_4$									15.72	15.85
Peimitidine	$C_{27}H_{44}O_3N$	75.29	75.38	75.06	10.30	9.89	10.04	3.25	3.37	3.51	
Peimitidine Hydrochloride	$C_{27}H_{44}O_3N \cdot HCl$							7.59	7.68	7.76	
Peimitidine Aurichloride	$C_{27}H_{44}O_3N \cdot HCl \cdot AuCl_3$									25.59	25.60

needles, m. p. 256°. Analysis confirms its composition as indicated in Table II.

Peimiphine, $C_{27}H_{46}O_3N$.—After filtering off the insoluble peimisine hydrochloride, the alcohol-soluble part of the crystalline deposit obtained above is evaporated to dryness and converted into its free base again by dissolving in water, making alkaline with sodium carbonate, and extracting with ether. The ethereal solution is dried and distilled and the residue neutralized with hydrobromic acid in a little alcohol. On addition of a sufficient quantity of ether, peimiphine hydrobromide crystallizes out in rhombic prisms. Peimiphine is liberated from its hydrobromide by action of sodium carbonate and recrystallizes from a mixture of acetone and petroleum ether. It forms hard prisms, m. p. 127°, $[\alpha]_D^{21}$ −69. It is easily soluble in chloroform and alcohol, but much less so in ether. Its hydrochloride, prepared by neutralizing the pure alkaloid with hydrochloric acid in acetone, forms orthorhombic prisms, m. p. 287°, and is easily soluble in water or alcohol. Its aurichloride is obtained as a yellow amorphous powder in a similar way as peimisine aurichloride. The composition of peimiphine is determined to be $C_{27}H_{46}O_3N$ according to the analytical results given in Table II.

Peimidine, $C_{27}H_{45}O_2N$.—After the removal of peimisine and peimiphine hydrochlorides as a crystalline deposit as described above, the mother liquor is distilled to almost dryness and the residue taken up with water and filtered. The aqueous solution is made alkaline with sodium carbonate and the free base extracted with ether. The ethereal solution is dried and distilled and the basic residue neutralized with concentrated hydrobromic acid in a little alcohol. On addition of ether, peimidine hydrobromide crystallizes out in rhombic prisms, m. p. over 300°. It is soluble in cold water with difficulty. Peimidine is obtained from its hydrobromide by action of sodium carbonate in an aqueous solution and extraction with chloroform. When crystallized pure from 95% alcohol, peimidine forms orthorhombic prisms, m. p. 222°, $[\alpha]_D^{26}$ −74°. It is only sparingly soluble in ether or acetone but easily soluble in chloroform or alcohol. Its hydrochloride forms rhombic prisms, m. p. 318°, easily soluble in alcohol or water. Its platinichloride is obtained as an orange amorphous powder by precipitation in aqueous solution in the presence of one drop of hydrochloric acid. The analytical data as given in Table II indicate its formula to be $C_{27}H_{45}O_2N$.

Peimitidine, $C_{27}H_{44}O_3N$.—The mother liquor of peimidine hydrobromide as described above is evaporated to dryness and the residue taken up with water and filtered. The free base is regenerated from the aqueous solution with sodium carbonate and extracted with ether. The ethereal solution is dried and distilled and the residue neutralized with concentrated hydrochloric acid in acetone. On long standing, peimitidine hydrochloride crystallizes out in rhomboid prisms. When recrystallized repeat-

edly from a mixture of alcohol and acetone, it has a melting point of 291° with decomposition, easily soluble in absolute alcohol or water. Peimitidine is obtained from its pure hydrochloride in a usual way and crystallizes from a mixture of acetone and petroleum ether in hard prisms, m. p. 188°, $[\alpha]_D^{20}$ −68°. It is easily soluble in most organic solvents, but much less so in ether. Its platinichloride is very soluble in water, but its aurichloride is easily obtained as an amorphous powder by precipitation in an aqueous solution. It has the composition of $C_{27}H_{44}O_3N$ as shown in Table II.

SUMMARY

From the mother liquor of peimine and peiminine, two principal alkaloids isolated from the Chinese drug, Pei-Mu, identified as *Fritillaria Roylei*, there have been isolated four other minor alkaloids to which the names peimisine, peimiphine, peimidine, and peimitidine are respectively assigned. Their formulas, melting points, and specific rotations are given in Table I. They are present in the drug only to the extent of 0.001 to 0.002 per cent. Peimisine easily forms an oxime, m. p. 196°.

REFERENCE

(1) Chou, T. Q., and Chu, T. T., *J. Am. Chem. Soc.*, **63**, 2936(1941).

The Comparative Chronic Toxicities of Fumaric, Tartaric, Oxalic, and Maleic Acids*

By O. GARTH FITZHUGH and ARTHUR A. NELSON

Data are given on the effects of the oral administration of fumaric, tartaric, oxalic, and maleic acids. The results of the study indicate a low degree of toxicity for fumaric, oxalic, and tartaric acids. The oxalic and tartaric acids were not toxic in concentrations as high as 1.2% and fumaric acid was toxic only at the 1.5% concentration. Maleic acid was toxic at concentrations of 0.5% or more.

THE SIMILAR laxative action produced by the salts of fumaric and tartaric acids suggested a comparative survey of the chronic toxicities of these acids. Because of a low acute toxicity of fumarates to animals and of their efficacy as compared with salts of tartaric acid, recent investigators (1–6) have proposed a substitution of fumarates for tartrates in laxative preparations. The nephropathic action of large acute doses of tartaric acid (7) does not appear to occur in animals given similar doses of fumaric acid (1); however, no lifetime study has been conducted with either of these acids. The presence of large amounts of oxalates in certain foods (8–12) and the isomerism of maleic and fumaric acids suggested the inclusion of oxalic and maleic acids in a two-year study of the chronic toxicities of these 4 dicarboxylic acids. The many experiments reported in the literature on the toxicity of oxalic acid have been either acute or short-term chronic experiments.

* Received Jan. 14, 1947, from the Division of Pharmacology, Food and Drug Administration, Federal Security Agency, Washington, D. C.

EXPERIMENTAL

Two experiments were conducted in which groups of weanling rats (twenty-one days) from our colony of Osborne–Mendel strain were started on diets containing one of the above-named dicarboxylic acids. A total of 420 rats was used in these experiments. In the first experiment 12 groups of 24 rats, equally divided between the sexes, were fed on diets containing 0.1, 0.5, 0.8, and 1.2% tartaric, fumaric, and oxalic acids, respectively. In a second experiment, in order to compare more closely the toxicities of fumaric and maleic acids, 6 groups of 12 male rats were fed on diets containing 0.5, 1.0, and 1.5% fumaric and maleic acids, respectively. There were 48 control animals for the first experiment and 12 con-

trols for the second experiment. Ground commercial rat biscuits with 1% added cod-liver oil served as the basic diet. The acids were mixed with the basic diet by means of a rotary batch mixer. All animals were kept in individual cages in a room with the temperature and humidity controlled for the duration of the experiment and were given free access to their respective diets and water. The weights of individual animals and their food consumption were determined at weekly intervals.

RESULTS

Since the two experiments were started at the same time and were part of the same study, they will be discussed together.

The Effect on Growth Rate and Food Consumption.—An inspection of Table I shows that all animals, with the exception of those on the two highest dosages of maleic acid, made a normal gain in weight during the first fifty-two weeks of the experiment. The slight differences between other groups of experimental animals and the controls were not significant ($p = 0.05$, or less, is significant). Individual growth curves of rats from the same litter and the composite growth curves of the several groups showed that there were some variations in the retarding effect of the acids both during the fast growing period and during the plateau period. Analyses, therefore, were made on the gains in weight for the first twelve weeks and for the first twenty-six weeks of the experiment. These analyses showed a significant difference between the mean gain in weight of the control animals and the mean gains in weight of the groups on 1% and 1.5% maleic acid, but showed no difference between the mean gain in weight of the controls and that of any group of fumaric, tartaric, or oxalic acids. Thus, of the 4 acids used in this study, only the maleic at concentrations of 1%, or more, retarded the growth rate of the rats.

When the data for the weekly food consumption of each group during the first twenty-six weeks and during the second twenty-six weeks of the experiment were analyzed, the differences between the control and experimental animals were not statistically significant.

The Effect on Mortality.—Statistical tests cannot be applied to the mean survival time since all surviving animals were sacrificed at the conclusion of two years on the experiment. Most of the deaths, except for a few animals distributed throughout all groups and killed because of middle ear disease, occurred during the second year of the experiment. The number of animals surviving at eighteen months and at two years, therefore, were selected as estimates of relative toxicity. At eighteen months the maleic acid at concentrations of 1% and 1.5% in the diet increased the mortality rate; however, the difference between the mortality rate of either group of experimental animals and that of the controls was not significant. At two years the maleic acid had increased the mortality rate significantly. All of the 12 experimental animals on 1.5% and 10 on each of the other concentrations of maleic acid died whereas 6 of the controls survived to the end of the experiment. Likewise at two years there were only 2 animals living on 1.5% fumaric acid; other dosages of fumaric acid had no effect on mortality rate. There was no significant difference between the mortality rate of the rats on any dosage level of tartaric, or oxalic acid and that of the controls.

TABLE I.—MEAN GAIN IN WEIGHT OF RATS FED DIETS CONTAINING FUMARIC, TARTARIC, OXALIC, OR MALEIC ACID FOR A YEAR

Substance	Dosage, %	No. of Animals	Sex	Mean Gain in Weight, Gm.	Standard Error of Mean, Gm.
Fumaric Acid	0.1	11	M	452.9	±19.0
	0.1	10	F	288.0	±10.0
	0.5	10	M	444.4	±20.7
	0.5	12	F	269.7	± 9.6
	0.8	9	M	429.6	±11.0
	0.8	12	F	265.2	± 5.7
	1.0	10	M	468.8	±25.9
	1.2	11	M	466.9	±14.6
	1.2	12	F	280.0	±10.2
	1.5	7	M	459.9	±23.2
Tartaric Acid	0.1	9	M	459.9	±16.7
	0.1	12	F	278.2	±10.6
	0.5	11	M	450.5	± 5.7
	0.5	10	F	286.3	± 6.1
	0.8	8	M	464.8	±16.7
	0.8	10	F	270.4	± 6.9
	1.2	9	M	437.8	±11.0
	1.2	11	F	258.0	± 7.5
Oxalic Acid	0.1	11	M	453.9	±19.1
	0.1	10	F	272.5	±11.1
	0.5	8	M	444.4	±20.7
	0.5	12	F	275.3	± 2.9
	0.8	10	M	464.2	±16.1
	0.8	11	F	259.8	± 3.4
	1.2	11	M	451.9	±15.6
	1.2	10	F	261.4	± 9.1
Maleic Acid	0.5	9	M	447.1	±15.5
	1.0	7	M	372.8	±20.6[a]
	1.5	9	M	274.3	±18.4[b]
Control		31	M	464.9	±13.4
		22	F	274.4	± 9.8

[a] $p < 0.05$.
[b] $p < 0.001$.

Pathology.—For reasons unconnected with these experiments a greater amount of microscopic pathological examination was done than would have been necessary to demonstrate the relatively minimal nature of the pathological changes produced by the acids. Because our nearly negative findings are based on a large amount of material, it is felt that they are worth presenting.

Autopsy was done on nearly all the rats used in these experiments, a few being omitted because of advanced post-mortem autolysis. As the animals were received in the pathology laboratory, alternate ones were taken for microscopic sectioning. In 213 rats, hematoxylin-eosin stained paraffin sections of

the following structures were routinely made: lung, heart, liver, spleen, pancreas, stomach, small intestine, kidney, adrenal, and testis. The following additional structures were sectioned frequently enough to make it reasonably certain that no changes in them were being caused by the acids: colon, bone marrow, leg bones, leg muscles, lymph nodes, uterus, ovary, thyroid, and parathyroid.

In all the foregoing structures, no difference could be seen between treated and control animals except the following. Slight periportal hypertrophy of the hepatic cells, coincident with slight centrolobular atrophy, was seen in 15 instances (11 in animals fed oxalic acid, 5 of these in the 0.1% group; none in controls). Enlarged and irregularly shaped epithelial cells in small to moderate numbers of renal tubules, generally the proximal convoluted, were seen in 4 rats on 1.5% and in 3 rats on 1.0% maleic acid, and in no other rats in the entire series. Rats fed 1.5% maleic acid had distinctly more atrophy of the liver and less focal calcification in large arteries than did any of the other groups, and, together with those receiving 1.5% fumaric acid, showed more atrophy of the testis than did any other group. Inanition is at least partly responsible for the atrophy of the liver and testis just mentioned. It may be of significance that 2 rats fed fumaric acid (1.0% and 0.5%, respectively) showed phlegmonous gastritis, a condition not seen in any other group.

With the few and relatively minor exceptions noted in the preceding paragraph, the gross and microscopic findings in this series of rats showed no difference between control and treated animals. Tumors and other more or less frequently occurring spontaneous diseases of older rats, such as periarteritis nodosa-like arterial lesions, bile duct proliferation in the liver, et cetera, showed no difference in incidence among the various animal groups.

SUMMARY

1. Toxic effects occurred in rats fed diets containing 0.5 per cent or more maleic acid and 1.5 per cent fumaric acid for two years. No significant toxic effect occurred in rats fed diets containing 1.2 per cent oxalic or tartaric acid.

2. Detailed microscopic pathological examination of 213 rats showed no major visceral damage from any of the acids at any level, and only relatively minor differences between control and treated animals.

3. Rats fed 1.5 per cent maleic acid showed more atrophy of the liver and (together with those on 1.5 per cent fumaric acid) of the testis than did the remaining groups. Inanition seems at least partly responsible for these differences.

4. Some of the rats fed oxalic acid showed slight periportal hypertrophy of the hepatic cells, and some of those fed maleic acid had atypical renal tubular ephithelial cells. Two rats fed fumaric acid had phlegmonous gastritis.

5. Maleic acid at a concentration of 1 per cent retarded the growth rate of rats.

6. All concentrations of maleic acid and the 1.5 per cent concentration of fumaric acid increased the mortality rate.

REFERENCES

(1) Bodansky, O., Gold, H., and Zahm, W., THIS JOURNAL, **31,** 1(1942).
(2) Gold, H., and Zahm, W., *ibid.*, **32,** 173(1943).
(3) Smyth, C. J., Brundage, R., Orten, J. M., and Smith, A. H., *Proc. Soc. Exptl., Biol. Med.*, **60,** 301(1945).
(4) Levey, S., Lasichak, A. G., Brimi, R., Orten, J. M., Smyth, C. J., and Smith, A. H., THIS JOURNAL, **35,** 298(1946).
(5) Krop, S., and Gold, H., *ibid.*, **34,** 86(1945).
(6) Locke, A., Locke, R. B., Schlesinger, H., and Carr, H., *ibid.*, **31,** 12(1942).
(7) Underhill, F. P., Leonard, C. S., Gross, E. G., and Johnson, F. C., *J. Pharmacol.*, **43,** 359(1931).
(8) Yeh, H. L., and Adolph, W. H., *Chinese J. Physiol.*, **13,** 209(1938).
(9) Kohman, E. F., *J. Nutrition*, **18,** 233(1939).
(10) Bose, N. C., *J. Indian Med. Assoc.*, **13,** 330(1944).
(11) Mitchell, H. H., and Smith, J. M., *J. Am. Med. Assoc.*, **129,** 871(1945).
(12) Hoover, A. A., and Karunairatnam, M. C., *Biochem J.*, **39,** 237(1945).

The Separation of Penicillins by Partition Chromatography*

By HENRY FISCHBACH, THOMAS E. EBLE, and MERLIN MUNDELL†

Penicillin K was successfully determined in a mixture of penicillins by means of partition chromatography in which silicic acid is the adsorbent, a buffer solution the immobile solvent, chloroform and ether the mobile solvents. There was sufficient resolution of the other penicillins to permit the isolation and purification of a penicillin F.

EARLY in 1946, considerable concern was expressed by those employing penicillin therapy as to the penicillin K content of commercial penicillin. Accumulated evidence indicated that commercial penicillin produced since May, 1944, had been less effective against early syphilis than that produced prior to this date (1, 2). Further, it was known that proportionately greater amounts of penicillin K at the expense of penicillin G had appeared in the commercial product. At that time this laboratory issued a preliminary report (3) on a chromatographic procedure which had been found satisfactory for determining penicillin K in the presence of other known penicillins. The purpose of this paper is to present a comprehensive report of this laboratory's experience with partition chromatography in the field of penicillin assay and isolation.

Initially, an attempt was made to resolve the penicillins by the classic type of adsorption chromatography. Norite, combinations of norite and celite, talc, combinations of talc and celite, alumina, and acid washed alumina were tried as adsorbents. A number of solvents and combinations of solvents were utilized but at best a 75% recovery of adsorbed penicillins was effected with an acid washed alumina column and 80% with a combination of 2 parts norite to 3 parts celite (545). No resolution was perceived in the former and only a slight degree of differentiation in the latter. Since we used an iodometric procedure (4) to assay the fractions eluted from the columns, those solvents which contained impurities reactive with iodine were purified before use.

Considering the variation in the hydrophilic properties of the penicillins along with available information on the use of buffered silica gel columns, it was evident that partition chromatography might quantitatively resolve penicillin K from a mixture of penicillins. The initial silicic acid adsorbent was prepared by the Gordon, Martin, and Synge technique (5). Early in this work it appeared that a 20% phosphate buffer solution of *p*H 6.4 was desirable as the immobile solvent. Considerable time was spent in comparing the results obtained by use of the above activated type of silicic acid and those available on the commercial market. Only two commercial products failed to give the desired results The aqueous extract of one was strongly basic and the other was so fine that the percolation rate was impractical even with applied pressure. It was found that the commercial products could be used if the adsorptive capacity of the particular batch of silicic acid was first determined.

EXPERIMENTAL

Preparation and Utilization of Chromatographic Column.—In this laboratory small amounts of buffered solution are added to a weighed amount of the silicic acid and the mixture thoroughly macerated in a mortar after each addition. A point is reached where the silicic acid after grinding remains "sticky" and tends to "ball." Note is made of the per cent buffer required to bring about this degree of saturation. In preparing a column, 5% to 10% less buffer than that which resulted in the above saturation is used. The column is formed from a slurry of buffered silicic acid in chloroform which had previously been washed with water to free it of alcohol. The chloroform slurry of silicic acid in the column is subjected to an approximate pressure of 80 mm. for the purpose of packing down the adsorbent. This packing by pressure is continued until the volume of the

* Received Feb. 13, 1947, from the Medical Division, Chemical Section, Food and Drug Administration, Federal Security Agency, Washington, D. C.

† The authors acknowledge the cooperation of the Division of Penicillin Control and Immunology for the bioassays, and of Mr. W. V. Eisenberg of the Division of Microbiology for the crystallographic data.

chloroform-silicic acid mixture remains relatively constant. Sufficient solvent is added as needed to keep the column from channeling and going dry. Care is taken to eliminate air pockets during the packing procedure, by rapidly rolling the column in a vertical position between the palms of the hands when necessary. For convenience the percolating rate used in this laboratory is adjusted to 25 ml./ten to fifteen minutes, although considerable latitude of the rate is permissible. Equally good results are obtained with percolating rates varying from 25 ml./seven minutes to 25 ml./thirty-five minutes. A 5-gallon carboy, equipped with manometer and rubber bulb, is used as a simple source of controlled air pressure.

There are a number of factors which make chloroform the mobile solvent of choice at this stage for placing the penicillins on the column. The free penicillins are extracted from the aqueous phase at *p*H 2 under which condition the partition coefficient of the penicillins between chloroform and water is very favorable. The low solubility of water in chloroform minimizes that amount of *p*H 2 buffer carried along in the organic phase. In addition, penicillin X remains in the aqueous phase thus eliminating one penicillin prior to chromatographic resolution. Lastly, chloroform affords a concentrated narrow band of activity at the top of the column, whereas the use of a solvent, such as ether, results in a broad band of activity even with a restricted volume, thus limiting the length of column available for the development of the chromatogram.

The aqueous mixture of penicillin salts is acidified to *p*H 2 at 0° and extracted with three successive portions of chloroform. The combined extracts are added to the prepared column. After the chloroform solution has passed into the adsorbent, a 25-ml. portion of chloroform is added. As the last of the chloroform passes into the adsorbent, the eluent is added and the penicillins resolved. Peroxide-free ether saturated with water is the most satisfactory eluent of those tried and, since the operations are conducted in a 10° room to minimize the destruction of penicillin, its volatility is not a serious drawback. By the above procedure, no activity is found in the eluates until ether begins to come off the column. Ordinarily, 25- to 50-ml. fractions are collected and assayed by the iodometric procedure for total penicillin. The chloroform or ether fractions are assayed directly without a previous extraction into an aqueous medium.

Data.—The preliminary studies were limited to commercial penicillin since some of the pure penicillins were not readily available. It was evident from the start that one band of activity traveled very rapidly through the column and was eluted within a relatively few 25-ml. fractions. Subsequent to this band there followed several fractions with no activity before appearance of another band of penicillin. From this point there was some overlapping of the fractions collected although distinct maxima and minima were evident. The determination of the subtilis-staphylococcus ratios and the crystallographic properties of the combined fractions of any one band established the sequence in which the penicillins were being eluted from the column. Penicillin K was the active component of the first band, followed by penicillin dihydro F, penicillin F, and penicillin G.

A small quantity of pure penicillin F was prepared by combining the ethereal fractions of the penicillin F band resulting from chromatographic resolution, removing the ether by reduced pressure in the presence of a buffer, extracting into chloroform at *p*H 2, re-extracting with sodium hydroxide to a *p*H of 7, lyophilizing and recrystallizing from acetone. Subtilis-staphylococcus ratios, crystallographic data, and infrared studies (6) indicate a penicillin F of very good quality.

When relatively pure penicillins K and dihydro F became available through the National Research Council, known amounts of penicillins K, dihydro F, F, and G were compounded and assayed as above. The known mixtures were prepared as follows: weighed quantities of the sodium salt of each penicillin were individually dissolved in ice water and extracted in chloroform at *p*H 2. Each chloroform extract was assayed separately and definite aliquots of each penicillin extract were mixed together. The mixture was added to the column and the subsequent recoveries were based on the assays of the original chloroform extracts.

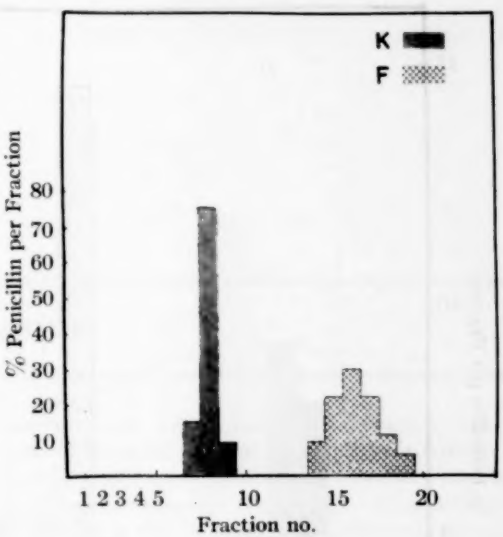


Figure 1

Figure 1 shows the separation of penicillins K and F on a column of Gordon, Martin, and Synge (5) activated silicic acid. In this study 101.3% of penicillin K and 101.6% penicillin F were recovered. Figure 2 represents the separation of penicillins K, F, dihydro F, and G on a column of commercial silicic acid. The recoveries in this case were 98.9% of penicillin K, 82.0% of penicillin dihydro F, 99.0%

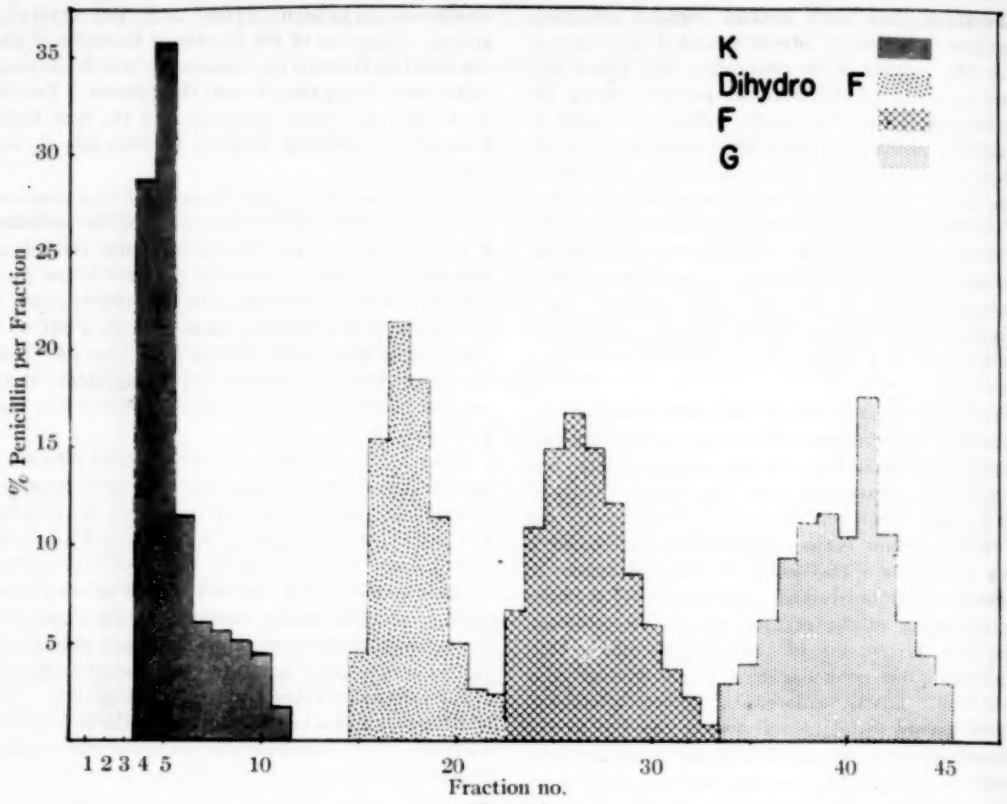


Figure 2

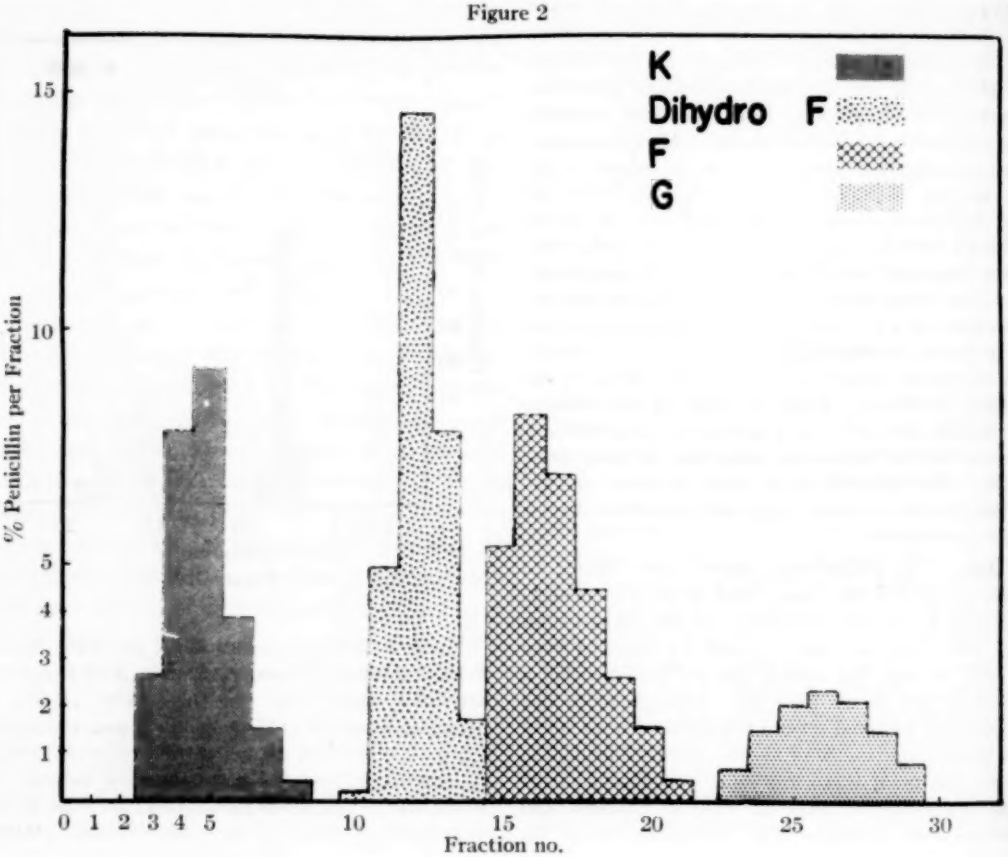


Figure 3

of penicillin F, and 98.3% of penicillin G. There is an unaccountable destruction of some of the penicillin dihydro F. Our results indicate that, at best, recoveries of between 80–85% of penicillin dihydro F can be obtained under the conditions necessary for optimum resolution of all the penicillins. Figure 3 shows the separation of a mixture of penicillins of unknown composition. This mixture was extracted directly into ether from a water solution at *p*H 2.0 and the extract placed on the column. The total recovery in this case was 94.0% of which 25.3% was penicillin K, 28.9% was penicillin dihydro F, 29.5% was penicillin F, and 10.3% was penicillin G. It can be inferred from other data that the inability to recover all of the penicillin was due to a partial destruction of penicillin dihydro F.

Since our primary interest had been the determination of penicillin K, it was advantageous that this penicillin should be the first to elute from the column and the one most completely separated under the above conditions. It was found that reproducible results, ±2%, for penicillin K were obtained under standardized conditions. This accuracy was obtained by arbitrarily fixing the dimensions of the columns, the pressure, the batch of silicic acid, the quantity of immobile solvent, and the quantities of mobile solvents. Having established the behavior of a mixture of penicillins under these conditions, it was possible to decide upon the quantity of eluate that would contain all of the penicillin K. Thus, only one fraction need be collected and assayed for penicillin K. In practice a subsequent fraction of 10 to 25 ml. was collected and assayed as a precautionary measure.

Previous experience with the iodometric assay for penicillin had been entirely on aqueous media. Consequently, the first assays entailed an extraction into water of each eluted fraction prior to the chemical determination. It was found, however, that the chloroform or ether fractions could be assayed directly by the iodometric procedure and the extraction step was eliminated.

SUMMARY

By means of partition chromatography, penicillin K was quantitatively determined in a mixture of penicillins. The same technique may be used for isolating and purifying the other penicillins. In this laboratory, a penicillin F of a high purity was so prepared.

REFERENCES

(1) Committee on Medical Research and U. S. Public Health Service, *J. Am. Med. Assoc.*, **131**, 265–71(1946).
(2) Committee on Medical Research, U. S. Public Health Service, and Food and Drug Administration, *ibid.*, pp. 271–75.
(3) Fischbach, H., Mundell, M., and Eble, T. E., *Science*, **104**, 84–85(1946).
(4) Mundell, M., Fischbach, H., and Eble, T. E., THIS JOURNAL, **35**, 373–78(1946).
(5) Gordon, A. H., Martin, A. J. P., and Synge, R. L. M., *Biochem. J.*, **37**, 79–86(1943).
(6) Barnes, R. Bowling, A Personal Communication, American Cyanamid Company, Stamford, Conn.

Book Review

Physical Constants of Hydrocarbons. Volume IV, Polynuclear Aromatic Hydrocarbons, by GUSTAV EGLOFF. Reinhold Publishing Corporation, New York, 1947. xi + 540 pp. 15 x 23 cm. Price $17.50.

For a review of Volume III of this series see THIS JOURNAL, **35**, 320(1946).

The source of most of the hydrocarbons described in this volume is coal tar and the commercial use is limited largely to dyestuffs. Many of these hydrocarbons, and more especially their derivatives, are of physiological interest. The carcinogenic hydrocarbons are in this group as are the hydrocarbon nuclei of the steroid compounds.

Like Volume III, this volume is a compilation of melting point, boiling point, and refractive index data of these polynuclear aromatic hydrocarbons. The same scheme of classification previously used is maintained throughout this volume. An additional advantage is the thorough documentation, several of the compounds having over fifty references cited.

In addition to the positive value of these data to the research workers, the gaps in the data focus attention on needs for further research.

Data of this type are very difficult to lay out, but the publishers have done an excellent job of setting up difficult material. All graphic formulas are sufficiently large to be comfortably read, and the publishers have used bold face type with a sense of discretion.—MELVIN W. GREEN.

Note

A Simple Color Reaction for Piperazine*

By ROBERT D. BARNARD

WHILE attempting to develop a gasometric method for the determination of uric acid, solutions of the latter in piperazine were treated with potassium ferricyanide[1] in the extraction chamber of a Van Slyke manometric apparatus (2). On shaking, a lilac color which reached an intensity peak in about five minutes and then faded, was noticed. Piperazine solution, itself, gave the same color on ferricyanide treatment provided metallic mercury was present. Since the reaction is not given by other diazines or by diketopiperazine, a color test for piperazine was developed on this basis, particularly when it was found that the color could be stabilized by buffering at *p*H 9.

Conduct of the Test.—To about 5 cc. of a solution suspected of containing piperazine which should contain more than one in ten thousand parts, add 10 drops of 5% $K_3Fe(CN)_6$ solution, or if the latter is found to be decolorized, the addition is continued, dropwise, until a lemon-yellow tint persists. A droplet of metallic mercury is now added, the tube stoppered and vigorously shaken so as to disperse the mercury.

In the presence of piperazine, a lilac color appears and fades after about ten minutes. If the solution to be tested has first been saturated with $NaHCO_3$ by addition of an excess of the solid substance, a rose-red color develops and persists indefinitely.

Interfering Substances and Conditions.—Substances reducing ferricyanide, e.g., uric acid, interfere; these are circumvented by addition of an excess over that required to oxidize such substances. Protein-containing fluids such as blood serum become turbid on treatment with the mercury; the pinkish color can, however, still be detected. The urine, particularly after piperazine administration, contains polyphenols which give a brownish or reddish color with the ferricyanide alone (catechol reaction). They may be removed from the test fluid by preliminary extraction of the acidified specimen with ether.

* Received Feb. 10, 1947, from the Laboratory Service, Halloran General Hospital, Staten Island, N. Y.

[1] To oxidize the uric acid to allantoin and recover the evolved carbon dioxide. The method failed as ferricyanide oxidation of uric acid does not yield allantoin directly as reported (1). No carbon dioxide is evolved except after rigorous alkai treatment. The primary oxidation product is probably uroxanic acid.

DISCUSSION

The test is rapid and specific and can be conducted with reagents common to any laboratory. It has been used in the attempt to demonstrate the presence of piperazine in the urine of human subjects after ingestion of 1 Gm. of the material. The results were negative which confirms the statement of Sollmann that piperazine is probably not excreted as such (3). In this connection an enhanced catechol reaction has been noticed in such urines, so it is possible that piperazine is excreted as a phenolic oxidation product.

The mechanism of the reaction is not clear, The ferricyanide is not reduced during its course, so the reaction product may be similar in structure to that of mercury fulminate and ferricyanide (4) whose color is almost identical. In the latter case, the mechanism can be surmised. Fulminate has a nitrile oxide structure (5) and the polar tenaille is sufficiently acute to permit penetration of the 4° coordination ring of the iron with polar linkage in the 3^2 orbit. Such compounds have been termed "heptacovalent" by Coryell and Stitt (6) but actually they are hexacoordinated unipolar covalent Piperazine is aresonant and stereochemically either of the imine nitrogens would constitute an acute polar tenaille which could enter such a covalent linkage. Whether this is the proper explanation of the colored compound, however, remains to be determined.

SUMMARY

A simple color test for the detection of piperazine, employing common laboratory reagents, is described. Urine specimens from normal subjects ingesting piperazine, were negative by this test.

REFERENCES

(1) Flatow, L., *Biochem. Z.*, **176,** 178(1926).
(2) Van Slyke, D. D., *J. Biol. Chem.*, **61,** 523(1924).
(3) Sollmann, T., "A Manual of Pharmacology," W. B. Saunders and Co., Philadelphia, 1937.
(4) Barnard, R. D., *J. Lab. Clin. Med.*, **24,** 649(1939).
(5) Pauling, L., and Hendricks, S. B., *J. Am. Chem. Soc.*, **48,** 641(1926).
(6) Coryell, C. D., and Stitt, F., *ibid.*, **62,** 2951(1940).